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(54) Title: METHODS AND COMPOSITIONS FOR MODULATION OF GROWTH RESPONSE

(57) Abstract

The present invention details methods for the treatment of cancer. In particular, it concerns the induction of apoptosis of cancer cells following treatment with c-Abl and p300, which control levels of wild-type p53. Also included are methods of combination therapy wherein cells lacking p53 are supplemented with c-Abl, p300 and wild-type p53 to induce an apoptotic responses.

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DESCRIPTION

METHODS AND COMPOSITIONS FOR MODULATION OF GROWTH RESPONSE

BACKGROUND OF THE INVENTION

The government may own rights in the present invention pursuant to grants from the National Institutes of Health.

1. Field of the Invention

The present invention relates generally to the fields of cancer therapy and control of cell proliferation. More particularly, it concerns the use of the c-Abl and p300 genes, either alone or in combination with p53, to induce programmed cell death or decreased cell proliferation.

2. Description of Related Art

Certain cancer treatment methods, including radiation therapy, involve damaging the DNA of the cancer cell. The cellular response to DNA damage includes activation of DNA repair, cell cycle arrest and lethality (Hall, 1988). The signaling events responsible for the regulation of these events, however, remain unclear.

Several checkpoints in cell cycle progression control growth in response to diverse positive and negative regulatory signals (Lau & Pardee, 1982). Ionizing radiation, for example, slows growth by inducing delays in G_1/S and G_2 phases of the cell cycle. The available evidence suggests that G_2 arrest in necessary for repair of DNA damage before entry into mitosis (Steinman *et al.*, 1991; Weinert & Hartwell, 1988). Genetic studies in *Saccharomyces cerevisiae* have demonstrated that the RAD9 protein controls G_2 arrest induced by DNA damage (Schiestl *et al.*, 1989; Murray, 1989). Mutants of the rad9 locus are unable to delay entry into mitosis following exposure to genotoxic agents and thereby replicate damaged DNA. Although the mammalian homolog of rad9 remains unidentified, other studies in various eukaryotic cells have demonstrated that entry into mitosis is regulated by a 34 kD

serine/threonine protein kinase, designated $p34^{cdc2}$ (Nurse, 1990; Pines & Hunter, 1989; Russell & Nurse, 1987).

Recent studies have shown that exposure of eukaryotic cells to ionizing radiation is associated with induction of certain early response genes that code for transcription factors. Members of the jun/fos and early growth response (EGR) gene families are activated by ionizing radiation (Sherman *et al.*, 1990; Datta *et al.*, 1992a). Expression and DNA binding of the nuclear factor kB (NF-kB) are also induced in irradiated cells (Brach *et al.*, 1991; Uckun *et al.*, 1992a). Other studies have shown that levels of the tumor suppressor p53 protein increase during X-ray-induced arrest of cells in G1 phase (Kastan *et al.*, 1991; 1992). The activation of these transcription factors presumably represents transduction of early nuclear signals to longer term changes in gene expression that constitute the response to irradiation.

Ionizing radiation also induces protein kinase C (PKC) and protein tyrosine kinase activities (Hallahan *et al.*, 1990; Uckun *et al.*, 1993). However, the specific kinases responsible for these activities and their substrates require further study. The interaction between radiation, cell signaling, phosphorylation and various other oncogenes and cellular protooncogenes has not been well studied to date.

Mitomycin C (MMC) is an antitumor antibiotic isolated from Streptomyces caespitosus that covalently binds to DNA (Tomasz *et al.*, 1988). This agent induces both monofunctional and bifunctional DNA lesions (Carrano *et al.*, 1979). Other studies have demonstrated that MMC stimulates the formation of hydroxyl radicals (Dusre *et al.*, 1989). Although the precise mechanism of action of this agent is unclear, MMC-induced cytotoxicity has been attributed to DNA alkylation and the formation of interstrand cross-links (Carrano *et al.*, 1979; Dusre *et al.*, 1989; Tomasz *et al.*, 1988). Treatment of mammalian cells with MMC is associated with inhibition of DNA synthesis and induction of sister-chromatid exchange (Carrano *et al.*, 1979). Previous work has demonstrated that MMC also enhances transcription of HIV-1 and collagenase promoter constructs transfected into HeLa cells (Stein *et al.*, 1989). These studies indicated that AP-1 is involved in MMC-induced activation of the collagenase enhancer. However, little is known about the effects of this agent on other signaling events.

Protein tyrosine phosphorylation contributes to the regulation of cell growth and differentiation. Protein tyrosine kinases can be divided into receptor-type and nonreceptor-type (Src-like) kinases (Cantley *et al.*, 1991; Hanks *et al.*, 1988; Bonni *et al.*, 1993; Lamer *et al.*, 1993; Ruff-Jamison *et al.*, 1993). Several protein tyrosine kinases have been purified from the cytosolic fractions of various tissues (Nakamura *et al.*, 1988; Wong & Goldberg, 1984; Zioncheck *et al.*, 1986).

The Src-like kinases, which can associate with receptors at the plasma membrane, induce rapid tyrosine phosphorylation and/or activation of effectors such as phospholipase C-γ1 (PLCγ1) (Carter *et al.*, 1991), PLCγ2 (Hempel *et al.*, 1992), mitogen-activated protein (MAP) kinase (Casillas *et al.*, 1991), GTPase activating protein (GAP) (Gold *et al.*, 1992a) and phosphatidylinositol 3-kinase (PI3-K) (Gold *et al.*, 1992b). Recent studies have demonstrated an increase in tyrosine phosphorylation following irradiation of B-lymphocyte precursors (Uckun *et al.*, 1993). Studies of $p59^{fyn}$, $p56/p53^{lyn}$, $p55^{blk}$ and $p56^{lck}$ activity demonstrated that these Src-family tyrosine kinases were not responsible for radiation-induced tyrosine phosphorylation (Uckun *et al.*, 1992a). These findings suggested that other protein tyrosine kinases, perhaps of the receptor-type, are involved in the response of cells to ionizing radiation.

SUMMARY OF THE INVENTION

It is, therefore, a goal of the present invention to exploit certain of the cell cycle pathways in order to circumvent aberrations in cellular growth regulation, thereby providing a method for the treatment of cancers. To this end, the inventors seek to exploit the p53-inducing properties of p300 and c-Abl.

Therefore, the present invention, in one embodiment, provides a method for increasing p53-mediated apoptosis in a tumor cell comprising the step of increasing the activity level of at least one of c-Abl and p300 in said tumor cell. The tumor cell may be derived from various tissues including brain, lung, liver, spleen, kidney, lymph node, small

intestine, blood cells, pancreas, colon, stomach, breast, endometrium, prostate, testicle, ovary, skin, head and neck, esophagus, bone marrow and blood tissue.

In a preferred embodiment, the activity levels of both c-Abl and p300 are increased in said tumor cell. Increasing activities may be accomplished (i) by providing at least one of a c-Abl polypeptide and a p300 polypeptide to said tumor cell in an amount effective to increase p53-mediated apoptosis, (ii) by providing at least one of a nucleic acid encoding c-Abl and a nucleic acid encoding p300 to said tumor cell, wherein said nucleic acid is operably linked to a promoter active in eukaryotic cells in an amount effective to increase p53-mediated apoptosis, or (iii) by providing an agent to said cell that increases the expression or stability of at least one of c-Abl and p300 in an amount effective to increase p53-mediated apoptosis.

Delivery in of polypeptides via liposomes is specifically contemplated. Delivery of nucleic acids, contained in viral expression vectors, via infection of target cells by encapsulated viral vectors also is contemplated. Various viral vectors include herpesvirus, adenovirus, vaccinia virus, retrovirus and adeno-associated virus.

Because p300 and c-Abl act though p53, it is important that the target cell express an active p53 molecule. Thus, the method may further comprise determining the p53 activity of the target cell. Where the target cell is defective in p53, and the method further comprises providing to said tumor cell a nucleic acid encoding a wild-type p53 operably linked to a promoter active in eukaryotic cells. Again, the p53 nucleic acid preferably is contained in an expression vector, and more preferably in a viral vector.

Combination therapies are advantageously employed with any of the foregoing embodiments. For example, target cells may be subjected, either before or after the methods described above, to ionizing radiation or to a chemotherapeutic agent. The ionizing radiation may be x-irradiation or γ-irradiation. The chemotherapeutic agent may be mitomycin C, etoposide, genistin, cisplatin, 5-FU, adriamycin, doxorubicin, actinomycin D, verapamil, nitrosourea, ara-C and camptothecin.

In another embodiment, there is provided a method for treating a patient having a tumor comprising the step of increasing the activity level of at least one of c-Abl and p300 in cells of said tumor. Again, the activity level may be increased by providing at least one of a c-Abl polypeptide and a p300 polypeptide to said tumor in an amount effective to increase p53-mediated apoptosis in cells thereof, by providing at least one of a nucleic acid encoding c-Abl and a nucleic acid encoding p300 to said tumor, wherein said nucleic acid is operably linked to a promoter active in eukaryotic cells in an amount effective to increase p53-mediated apoptosis in cells thereof, or by providing an agent to said tumor that increases the expression or stability of at least one of c-Abl and p300 in an amount effective to increase p53-mediated apoptosis in cells thereof. The polypeptide, nucleic acid or agent may be provided via intratumoral injection.

Again, the p53 function of a cell is an important aspect of the invention. Therefore, the method may further comprising screening the tumor cells to determine the p53 status thereof. In addition, if the tumor cells lack p53 function, the method may further comprise providing to said tumor cells a nucleic acid encoding a wild-type p53 operably linked to a promoter active in eukaryotic cells. The method may further take advantage of combined therapies, for example, by additionally treating the tumor with ionizing radiation or a chemotherapeutic agent.

In still yet another embodiment, there is provided a method of screening a candidate substance for p53-stimulatory activity comprising the steps of (i) providing a eukaryotic cell expressing a functional p300 polypeptide; (ii) contacting said cell with said candidate substance; and (iii) determining the effect of said candidate substance on the p300 level of said cell, wherein an increase in the p300 level in said cell, as compared to an untreated cell, indicates that said candidate substance increases p53 activity. A similar method is provided where the effect of the candidate substance on c-Abl is determined.

The p300 or c-Abl level may be measured by Western blot or ELISA. Where the candidate substance is a nucleic acid encoding a polypeptide operably linked to a promoter active in eukaryotic cells, contacting comprises transferring said nucleic acid

into said cell. Methods of transferring include transfection, lipofection, protoplast fusion, bombardment, electroporation or viral infection.

In still yet other embodiments, there are provided (i) a use of at least one of a p300 and a c-Abl polypeptide for the preparation of a pharmaceutical composition effective to increase p53-mediated apoptosis in a cell, (ii) a use of at least one of a nucleic acid encoding a p300 polypeptide and a nucleic acid encoding a c-Abl polypeptide for the preparation of a pharmaceutical composition effective to increase apoptosis in a cell, (iii) a use of a p300 polypeptide or gene coding therefor for the preparation of a pharmaceutical composition for the treatment of cancer, and (iv) a use of a p300 polypeptide or gene coding therefor for the preparation of a pharmaceutical composition for the treatment of cancer.

Still further embodiments of the present invention are kits for use in killing malignant cells or reducing their growth, as may be formulated into therapeutic kits for use in cancer treatment. The kits of the invention will generally comprise, in suitable container means, pharmaceutical formulations of a p53 polypeptides or gene construct, c-Abl polypeptides or gene constructs and p300 polypeptides or gene constructs. These agents may be present within a single container, or these components may be provided in distinct or separate container means. Classical chemotherapeutic pharmaceutical preparations also are contemplated for use with the therapeutic compositions of the present invention.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein:

FIGS. 1A-C. Overexpression of kinase active c-Abl downregulates Cdk activity. FIG. 1A, MCF-7 cells were transfected with 2 μg p53-enhancer-luciferase plasmid (mdm2NA-Luc) and; (1) 8 μg control vector pSRaMSVtkNeo; (2) 5 and 8 μg c-abl vector, (3) 5 and 8 μg c-Aabl (K-R) vector; and (4) 5 and 8 μg c-ablΔPro[4] vector. Cells were also transfected with 2 μg SV40-promoter-luciferase plasmid (pGL2-control vector, Promega) and 8 μg c-Abl vector. Luciferase activity was measured and normalized for protein concentration to that for control vector, D, MCF-7 cells were transfected with 8 μg control, c-abl, c-abl (K-R) of c-ablΔPro[4] vector. FIG. 1B, Cell lysates were immunoblotted with and-p21 (WAF1, Ab-1: Oncogene Solane) (top) and and-PCNA (PC10: Santa Cruz Biotechnology) (bottom). FIG. 1C, Cell lysates from the transient transfectants were imunoprecipitated (IP) with anti-Cdk2 (M2, Santa Cruz Biotechnology), followed by histone HI kinase assay (top) or immunoblotting (IB) with anti-Cdk2 (bottom).

FIGS. 2A-D. c-Abl kinase activity regulates irradiation (IR)-induced inhibition of Cdk2. FIG. 2A, MCF-7 cells stably transfected with null pSR vector or *c-abl* (K-R) were treated with 5 Gy IR and collected after 3h. Nuclei were isolated and nuclear proteins immunoprecipitated with anti-Abl (Ab-3; Oncogene Science) as described. Immune-complex kinase was analyzed using a glutathione S transferase (GST)-Crk (120-225) fusion protein as substrate (top). And Abl immunoprecipitates were also analyzed by immunoblotting with anti-Abl (bottom). FIG. 2B, Lysates were immunoblotted with anti-Abl, anti-p53 (Ab-6; Oncogene Science), anti-p21, anti-GADD45 (AT-26; Santa Cruz Biotechnology) and anti-c-Myc (9E10; Santa Cruz Biotechnology). FIG. 2C, Lysates were immunoprecipitated with anti-Cdk2. Precipitates were analyzed by immunoblotting

with anti-p21 and anti-Cdk2. FIG. 2D, Anti-Cdk2 immunoprecipitates were analyzed by histone H1 kinase assay (top) or immunoblotting with anti-Cdk2 (bottom).

FIGS. 3A-D. c-Abl kinase activity regulates IR-induced growth arrest. FIG. 3A, Representative two-dimensional FACS analysis of MCF-7/pSR and MCF-7/c-Abl (K-R) cells after exposure to 0 or 5 Gy IR. Synchronized cells were irradiated and percentage of cells in S phase was assessed at 24h.. Boxes labeled R1, R2 and R3 represent S, G1 and G2/M phase cells, respectively. FIG. 3B, Percentage of cells entering S phase after IR relative to control unirradiated cells. Results are expressed as the mean ± s.e. of 6 experiments for each of two (a,b) independently selected clones. FIG. 3C, C57BL6 wild-type (*abl*$^{+/+}$) and *abl*$^{-/-}$ MEFs were exposed to 0 or 5 Gy and collected at 3 h. Cell lysates were immunoblotted with anti-Abl and anti-PCNA antibodies. FIG. 3D, *abl*$^{+/+}$) and *abl*$^{-/-}$ MEFs were exposed to 0.5 or 20 Gy IR. Results (mean±s.e. of 5 experiments) are expressed as the percentage of cells entering S phase after IR relative to unirradiated cells.

FIGS. 4A-D. c-Abl kinase downregulates Cdk2 activity by a p53-dependent, p21-independent mechanism. FIG. 4A, Lysates were immunoprecipitated with anti-Abl (left) or anti-p53 (right) and the precipitates analyzed by immunoblotting with the indicated antibodies. FIG. 4B, MEFs (p59$^{-/-}$, p53$^{+/+}$)[11] were transfected with the *c-abl* or *c-abl* (K-R) vectors and collected at 48 h. FIG. 4C, MEFs (*abl*$^{+/+}$, *abl*$^{-/-}$)[11] were exposed to D or 5 Gy and collected at the indicated times. FIG. 4D, MEFs (*p21*$^{-/-}$, *p21*$^{+/+}$ were transfected with the c-Abl or c-Abl (K-R) vectors and collected at 48 h. Cell lysates were immunoprecipitated with anti-Cdk2 and the precipitates assayed for histone H1 kinase activity (top) or immunoblotted with anti-Cdk2 (bottom) as described for FIGS. 1A-C..

FIGS. 5. Effects of MMS on down-regulation of Cdk2 activity and growth arrest in Abl-deficient cells. Percentage of cells in S phase at 9 h of MMS treatment relative to untreated cells. Results are expressed as the mean = S.E. of six studies.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Accumulation of wild-type p53 protein results in induction of two pathways, cell cycle arrest and programmed cell death or apoptosis. A mutation in the p53 gene often inactivates these normal functions of the p53 gene and contribute to the malignant progression by gain of transforming potentiality. As the understanding of p53 biology continues to grow, the promise grows of applying this knowledge to cancer therapy. The introduction of wild-type p53 in a wide variety of p53 mutated cells using viral delivery systems resulted in increased expression of wild-type p53 and suppressed the malignant phenotype which holds hope for the gene replacement strategy as a biological therapy. The sustained high level of wild-type p53 expression leads to cell death. In other words, any means by which selective high level of p53 expression in the cancer cells is achieved could have potential implication in therapy of the disease.

The c-Abl protein tyrosine kinase is activated by certain DNA-damaging agents, and its overexpression causes arrest in the G1 phase of the cell cycle by the tumor-suppressor protein p53 (Sawyers *et al.*, 1994; Mattioni *et al.*, 1995; Goga *et al.*, 1995). The present investigators studied the role of c-Abl in growth arrest induced by DNA damage. Transient transfection experiments using wild-type or inactivated c-Abl show that both induce expression of p21, an effector of p53, but only wild-type c-Abl down regulates the activity of the cyclin-dependent kinase Cdk2 and causes growth arrest.

Exposure to ionizing radiation of cells that stably express active or inactive c-Abl is associated with induction of c-Abl/p53 complexes and p21 expression. However, cells expressing the dominant-negative c-Abl mutant and cells lacking the c-*abl* gene are impaired in their ability to down-regulate Cdk2 or undergo G1 arrest in response to ionizing radiation. It also is shown that expression of c-Abl kinase in $p21^{-/-}$, but not in $p53^{-/-}$, cells results in down regulation of Cdk2. These results suggest that c-Abl kinase contributes to the regulation of growth arrest induced by ionizing radiation by a p53-dependent, p21-independent mechanism.

The present inventors also have demonstrated that c-Abl associates with p53 in the cellular response to the antimetabolite 1-β-D-arabinofuranosylcytosine (Ara-C) and the alkylating agent methyl methanesulfonate (MMS). Ara-C misincorporates into cellular DNA (Kufe *et al.*, 1980; Major *et al.*, 1981) and blocks replication by site-specific termination of DNA strands (Kufe *et al.*, 1984; Ohno *et al.*, 1988; Townsend and Cheng, 1987). MMS is a monofunctional alkylating agent that induces DNA base damage and strand breaks (Schwartz, 1986). The results indicate that the c-Abl kinase function is involved in the growth arrest response induced by these and certain other genotoxic agents.

p300 is a nuclear protein originally identified for its ability to interact with adenovirus E1A protein. It is a regulator of transcription and interacts with a variety of cellular as well as viral proteins. p300 possesses histone acetyltransferase activity resulting from either intrinsic activity and/or from an assocaited protein, P/CAF. Bannister *et al.*, 1996, Ogryzko *et al.*, 1996, Yang *et al.*, 1996. The acetylation of histones in thought to be involved in destabilization and restructuring of nucleosomes, which is likely a crucial event for accessibility of transcription factors to DNA templates. These studies thus suggest that p300 participates in the transcription process by scaffolding different classes of transcriptional regulators on specific chromatin domains.

A role for p300 in control of cellular growth has been proposed on the basis of the functional behavior of adenovirus E1A and SV40 Tag proteins, which lose the ability to bind p300. Moran, 1993, Avantaggiati *et al.*, 1996; Eckner *et al.*, 1996a. Such mutants are defective in the induction of cellular DNA synthesis and, in several instances, of transformation. In addition, p300 is required for the activation of muscle-specific genes and for cell cycle arrest during differentiation of muscle cells. Eckner *et al.*, 1996b; Puri *et al.*, 1997. Moreover, mutations or translocation of the p300 gene has been described in human tumors. Borrow *et al.*, 1996; Muraoka *et al.*, 1996. p300 mutations in colorectal carcinomas are somatic and coupled to deletion of the second allele of the gene, suggesting that p300 is consequently inactivated. It also is induced upon a cell's exposure

to radiation. On the basis of this evidence, p300 is envisioned to be a negative regulator of cell growth.

Because p300 and p53 both are implicated in regulation of transcription, the possibility has been raised that p53 and p300 interact in this endeavor. It now has been demonstrated tha tp53 and p300 interact to form a specific protein complex in a variety of different cell types. The binding of p300 to p53 apparently results in activation of p53-dependent promoters, inhibtion of AP-1 regulated enhancers, G1 arrest and apoptosis. A fragment of p300 (p300 1514-1922) contains the regions required for interaction with p53 and can prevent p53 transcriptional activitation. It also has been determined that p300 interacts with c-Abl. c-Abl apparently phosphorylates p300 at one or more tyrosine residues, thereby activating p300 to interact with p53, thereby inducing the aforementioned p53-dependent actions.

A fundamental role for p300 has been established in the activation of p53. Physical interaction of these two proteins has been documented. p300 apparently is responsible for several p53-mediated functions, such as inhibition of AP1-regulated enhancers, transcriptional activation, apoptosis and cell cycle progression. The present inventors have determined that p300 is activated by c-Abl. More specifically, p300 is phosphorylated by a tyrosine kinase function of c-Abl. This phosphorylation causes p300 interaction with p53, and the aforementioned activities.

The related activities of these three molecules clearly impact on the ability of a cell, normal or abnormal, to progress through its cell cycle and proliferate. These functions, along with normal cell senescence, represent key points at which malignant cells might be attacked in a therapeutic treatment.

A. The Present Invention

The present invention exploits signaling pathways that are involved in the correction of DNA damage, in cell cycle progression and in apoptosis. In particular, the methods involve the use of the c-Abl, p300 and p53 gene products to effect cellular responses to various external stimuli.

In more particular aspects, the present invention provides a means of increasing the levels of wild-type p53 expression in tumor cells, thereby allowing for an increased apoptosis or reduction in cellular proliferation. The inventors have discovered that c-Abl and p300 can increase the expression of wild-type p53. In using this discovery in cell therapy, cancers that express normal p53 can be treated with c-Abl genes or the c-Abl gene product, or with p300 or the corresponding p300 gene. Secondly, the compositions of the present invention can be used to augment conventional gene therapy, where wild-type p53 is introduced into tumor cells concomitantly with c-Abl and/or p300 to increase the level of p53 so that programmed cell death is triggered. A third way in which the present invention could be employed is in combination therapy, where gene therapy is used in combination with conventional chemotherapy and radiotherapy, and the c-Abl and p300 gene products are used to increase wild-type p53 expression thereby inducing programmed cell death.

Therefore, the present application is drawn, in one embodiment, to methods of screening cells for their ability to express wild-type p53 protein expression of cancer cells and to concomitantly induce apoptosis in said cells. To augment the expression of p53, one would then add the c-Abl gene product and/or p300 to a cell. If wild-type p53 is lacking in a cell, wild-type p53 and c-Abl are administered to a cell in combination to induce the apoptotic response.

"Effective amounts" are those amounts of compositions effective at reproducibly increasing wild-type p53 expression in cancer cells in comparison to their normal levels. Compounds that achieve significant appropriate changes in activity will be used. A significant increase in wild-type p53 expression, *e.g.*, as measured using for example, Western blot analysis, are represented by an increase in wild-type p53 levels of at least about 30%-40%, and most preferably, by increases of at least about 50%, with higher values of course being possible. Assays that measure p53 content and expression in cells are well known in the art and may be conducted *in vitro* or *in vivo*.

Alternatively, it may be desirable simply to measure inhibition of growth of cancer cells, for example, by measuring growth according to the MTT assay. A significant

inhibition in growth is represented by decreases of at least about 30%-40% as compared to uninhibited, and most preferably, of at least about 50%, with more significant decreases also being possible. Growth assays as measured by the MTT assay are well known in the art. Assays may be conducted as described by Mosmann *et al.*, 1983; Rubinstein *et al.*, 1990 (incorporated herein by reference). Therefore, if a candidate substance exhibited inhibition of growth of cancer cells in this type of study, it would likely be a suitable compound for use in the present invention.

The more preferred approach will involve the preparation of vectors that incorporate nucleic acid sequences that encode the c-Abl and p300 sequences. It is contemplated that these vectors may either be transiently incorporated into the host cell, or may be stably integrated into the host cell genome. This expression preferably occurs in a mammalian cell, and even more preferably, the mammalian cell is a human cell. Examples of suitable vectors for use within the scope of the present invention include, but are not limited to, adenovirus, adeno-associated virus, retrovirus or herpes simplex virus 1.

Therefore, in certain aspects, the present invention contemplates the preparation of nucleic acid molecules that comprise a coding region that contains regions complementary to and capable of hybridizing with a c-Abl gene sequence. Generally, the preferred nucleic acid molecules will be DNA sequences arranged in a vector, such as a virus or plasmid, and positioned under the control of an appropriate promoter. However, as previously set forth, the antisense RNA molecule may itself be an appropriate nucleic acid, such as retrovirus RNA into which the appropriate coding sequences have been incorporated. Moreover, the nucleic acids may be introduced into cells by means of liposomes, or the like.

To kill malignant cells or reduce their growth, using the methods and compositions of the present invention, one would generally contact a "target" cell with a c-Abl and/or p300 construct in a combined amount effective to kill the cell. This process may involve contacting the cells with the p53 construct and c-Abl/p300 construct(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the p53 construct and the other

composition includes the c-Abl construct. Naturally, it is also envisioned that the target cell, where lacking a functional p53 molecule, may be first exposed to a p53 expression construct and then contacted with a c-Abl or p300 construct, or *vice versa*.

The terms "contacted" and "exposed", when applied to a cell, are used herein to describe the process by which a p53 or c-Abl construct are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or reduced cell growth, either one or both agents are delivered to a cell in a combined amount effective to kill the cell, *i.e.*, to induce programmed cell death or apoptosis. The terms, "killing," "programmed cell death" and "apoptosis" are used interchangeably in the present text to describe a series of intracellular events that lead to target cell death. Reduced cell growth refers to a lower rate of cellular proliferation than observed for the untreated cells.

The specific embodiments of the present invention, as set out above, are explained in greater detail as follows.

B. p53 As a Tumor Suppressor

p53 is currently recognized as a tumor suppressor gene (Montenarh, 1992). High levels have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses, including SV40. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is already documented to be the most frequently-mutated gene in common human cancers (Mercer, 1992). It is mutated in over 50% of human NSCLC (Hollestein *et al.*, 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 393-amino-acid phosphoprotein that can form complexes with host proteins such as large-T antigen and E1B. The protein is found in normal tissues and cells, but at concentrations which are minute by comparison with transformed cells or tumor tissue. Interestingly, wild-type p53 appears to be important in regulating cell growth and division. Overexpression of wild-type p53 has been shown in some cases to be anti-proliferative in human tumor cell lines. Thus p53 can act as a negative regulator of cell growth (Weinberg, 1991) and may directly suppress uncontrolled cell

growth or indirectly activate genes that suppress this growth. Thus, absence or inactivation of wild type p53 may contribute to transformation. However, some studies indicate that the presence of mutant p53 may be necessary for full expression of the transforming potential of the gene.

Wild-type p53 is recognized as an important growth regulator in many cell types. Mis-sense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. A single genetic change prompted by point mutations can create carcinogenic p53. Unlike other oncogenes, however, p53 point mutations are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Casey and colleagues have reported that transfection of DNA encoding wild-type p53 into two human breast cancer cell lines restores growth suppression control in such cells (Casey *et al.*, 1991). A similar effect has also been demonstrated on transfection of wild-type, but not mutant, p53 into human lung cancer cell lines (Takahasi *et al.*, 1992). The p53 appears dominant over the mutant gene and will select against proliferation when transfected into cells with the mutant gene. Normal expression of the transfected p53 does not affect the growth of cells with endogenous p53. Thus, such constructs might be taken up by normal cells without adverse effects.

It is thus possible that the treatment of p53-associated cancers with wild type p53 may reduce the number of malignant cells. However, studies such as those described above are far from achieving such a goal, not least because DNA transfection cannot be employed to introduce DNA into cancer cells within a patients' body.

C. Treatment of p53 Positive Cancers Using c-Abl and p300

A patient presenting a p53-positive tumor that expresses wild-type p53 will be treated with c-Abl and/or p300 based therapies. In such a case, the p53 status of the tumor cells will be determined using any conventional methods, examples of which are described below. Patients may, but need not, have received previous chemo-, radio- or gene therapies. Optimally, patients will have adequate bone marrow function (defined as peripheral absolute granulocyte count of > 2,000/mm^3 and platelet count of 100,000/mm^3), adequate liver function (bilirubin $\leq$ 1.5 mg/dl) and adequate renal function (creatinine < 1.5 mg/dl).

The patient will be treated with a pharmaceutically acceptable form of the c-Abl and/or p300 gene products, or will be treated by gene therapy techniques that will allow the *in vivo* expression of the c-Abl or p300 gene products. Alternatively, the patient may be treated with a substance that augments the expressionon c-Abl or p300 normally produced by the target cell. Treatment regimens normally last between about 3 and 6 weeks, and involve repeat dosings. In order to know whether a cell will respond to provision of c-Abl and/or p300, it must first be determined whether the cell expresses an active p53 molecule. Thus, screening for p53 expression is an important first step.

1. Determination of p53 Status of Target Cells

A wide variety of detection methods can be employed in the present invention to detect the p53 status of a cell. There are numerous antibodies to the p53 protein, hence any assay that utilizes antibodies for detection, for example, ELISAs, Western Blotting, immunoassay techniques etc. Alternatively, assays that employ nucleotide probes may be used to identify the presence/absence/status of p53, for example, Southern blotting, Northern blotting or PCR techniques. All the above techniques are well known to one of skill in the art and could be utilized in the present invention without undue experimentation.

i. ELISAs, Immunoassay and Immunohistological Assay

Immunoassays encompassed by the present invention include, but are not limited to those described in U.S. Patent No. 4,367,110 (double monoclonal antibody sandwich

assay) and U.S. Patent No. 4,452,901 (Western blot). Other assays include immunoprecipitation of labeled ligands and immunocytochemistry, both *in vitro* and *in vivo*.

Immunoassays, in their most simple and direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful.

In one exemplary ELISA, anti-p53 antibodies are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition suspected of containing the desired antigen, such as a clinical sample, is added to the wells. After binding and washing to remove non-specifically bound immune complexes, the bound antigen may be detected. Detection is generally achieved by the addition of another antibody, specific for the desired antigen, that is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA". Detection may also be achieved by the addition of a second antibody specific for the desired antigen, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

Varaitions of ELISA techniques are know to those of skill in the art. In one such variation, the samples suspected of containing the desired antigen are immobilized onto the well surface and then contacted with the antibodies of the invention. After binding and appropriate washing, the bound immune complexes are detected. Where the initial antigen specific antibodies are linked to a detectable label, the immune complexes may be detected directly. Again, the immune complexes may be detected using a second antibody that has binding affinity for the first antigen specific antibody, with the second antibody being linked to a detectable label.

Competition ELISAs are also possible in which test samples compete for binding with known amounts of labeled antigens or antibodies. The amount of reactive species in the unknown sample is determined by mixing the sample with the known labeled species

before or during incubation with coated wells. The presence of reactive species in the sample acts to reduce the amount of labeled species available for binding to the well and thus reduces the ultimate signal.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. These are described as below.

Antigen or antibodies may also be linked to a solid support, such as in the form of plate, beads, dipstick, membrane or column matrix, and the sample to be analyzed applied to the immobilized antigen or antibody. In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

In ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of the antigen or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the clinical or biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate

buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background.

The suitable conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25° to 27°C, or may be overnight at about 4°C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. Washing often includes washing with a solution of PBS/Tween, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immune complex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immune complex formation, *e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween.

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid [ABTS] and H_2O_2, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra spectrophotometer.

Alternatively, the label may be a chemiluminescent one. The use of such labels is described in U.S. Patent Nos. 5,310,687, 5,238,808 and 5,221,605.

Assays for the p53 status of the cell can determine normal/abnormal tissue distribution for diagnostic purposes. Methods for *in vitro* and *in situ* analysis are well known and involve assessing binding of antigen-specific antibodies to tissues, cells or cell extracts. These are conventional techniques well within the grasp of those skilled in the art. For example, the antibodies to p53 may be used in conjunction with both fresh-frozen and formalin-fixed, paraffin-embedded tissue blocks prepared for study by immunohistochemistry (IHC). Each tissue block may consist of 50 mg of residual "pulverized" tumor. The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, *e.g.*, in breast cancer, and is well known to those of skill in the art. (Abbondanzo *et al.*, 1990; Allred *et al.*, 1990; Brown *et al.*, 1990)

Briefly, frozen-sections may be prepared by rehydrating 50 ng of frozen pulverized tumor at room temperature in PBS in small plastic capsules; pelleting the particles by centrifugation; resuspending them in a viscous embedding medium (OCT); inverting the capsule and pelleting again by centrifugation; snap-freezing in -70°C isopentane; cutting the plastic capsule and removing the frozen cylinder of tissue; securing the tissue cylinder on a cryostat microtome chuck; and cutting 25-50 serial sections containing an average of about 500 remarkably intact tumor cells.

Permanent-sections may be prepared by a similar method involving rehydration of the 50 mg sample in a plastic microfuge tube; pelleting; resuspending in 10% formalin for 4 hours fixation; washing/pelleting; resuspending in warm 2.5% agar; pelleting; cooling in ice water to harden the agar; removing the tissue/agar block from the tube; infiltrating and embedding the block in paraffin; and cutting up to 50 serial permanent sections.

ii. Detection of p53 Nucleic Acids

In some situations, it may be preferable to look for p53 nucleic acids, in particular, for DNA's, cDNA's and mRNA's encoding p53. Nucleic acids may be isolated from cells according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where mRNA is used, it may be

desired to convert the mRNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA; in another, it is poly-A RNA. Normally, the nucleic acid is amplified.

Depending on the format, the specific nucleic acid of interest is identified in the sample directly using amplification or with a second, known nucleic acid following amplification. Next, the identified product is detected. In certain applications, the detection may be performed by visual means (*e.g.*, ethidium bromide staining of a gel). Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals.

Various types of defects are to be identified. Thus, "alterations" should be read as including deletions, insertions, point mutations and duplications. Point mutations result in stop codons, frameshift mutations or amino acid substitutions. Somatic mutations are those occurring in non-germline tissues. Germ-line tissue can occur in any tissue and are inherited. Mutations in and outside the coding region also may affect the amount of p53 produced, both by altering the transcription of the gene or in destabilizing or otherwise altering the processing of either the transcript (mRNA) or protein.

A variety of different assays are contemplated in this regard, including but not limited to, fluorescent *in situ* hybridization (FISH), direct DNA sequencing, PFGE analysis, Southern or Northern blotting, single-stranded conformation analysis (SSCA), RNAse protection assay, allele-specific oligonucleotide (ASO), dot blot analysis, denaturing gradient gel electrophoresis, RFLP and PCR-SSCP.

The term primer, as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred. Probes are defined differently, although they may act as primers. Probes, while perhaps capable of priming,

are designed to binding to the target DNA or RNA and need not be used in an amplification process.

In preferred embodiments, the probes or primers are labeled with radioactive species (^{32}P, ^{14}C, ^{35}S, ^{3}H, or other label), with a fluorophore (rhodamine, fluorescein) or a chemillumiscent (luciferase).

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR™) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, each of which is incorporated herein by reference in its entirety.

Briefly, in PCR, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

A reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in WO 90/07641 filed December 21, 1990. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPO No. 320 308, incorporated herein by reference in its entirety. In LCR, two

complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention, Walker *et al.*, (1992).

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference in their entirety). In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNA's are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, EPO No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer

oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, M.A., In: *PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS*, Academic Press, N.Y., 1990; herein incorporated by reference in its entirety).

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention. Wu *et al.*, (1989), incorporated herein by reference in its entirety.

Blotting techniques are well known to those of skill in the art. Southern blotting involves the use of DNA as a target, whereas Northern blotting involves the use of RNA

as a target. Each provide different types of information, although cDNA blotting is analogous, in many aspects, to blotting or RNA species.

Briefly, a probe is used to target a DNA or RNA species that has been immobilized on a suitable matrix, often a filter of nitrocellulose. The different species should be spatially separated to facilitate analysis. This often is accomplished by gel electrophoresis of nucleic acid species followed by "blotting" on to the filter.

Subsequently, the blotted target is incubated with a probe (usually labeled) under conditions that promote denaturation and rehybridization. Because the probe is designed to base pair with the target, the probe will binding a portion of the target sequence under renaturing conditions. Unbound probe is then removed, and detection is accomplished as described above.

It normally is desirable, at one stage or another, to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods. See Sambrook *et al.*, 1989.

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography.

Products may be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by a labeled probe. The techniques involved are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. For example, chromophore or radiolabel probes or primers identify the target during or following amplification.

One example of the foregoing is described in U.S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

In addition, the amplification products described above may be subjected to sequence analysis to identify specific kinds of variations using standard sequence analysis techniques. Within certain methods, exhaustive analysis of genes is carried out by sequence analysis using primer sets designed for optimal sequencing (Pignon *et al*, 1994). The present invention provides methods by which any or all of these types of analyses may be used. Using the sequences disclosed herein, oligonucleotide primers may be designed to permit the amplification of sequences throughout the p53 gene that may then be analyzed by direct sequencing.

All the essential materials and reagents required for detecting and sequencing p53 and variants thereof may be assembled together in a kit. This generally will comprise preselected primers and probes. Also included may be enzymes suitable for amplifying nucleic acids including various polymerases (RT, Taq, Sequenase™, *etc.*), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification.

Such kits also generally will comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each primer or probe.

Specifically contemplated by the present inventors are chip-based DNA technologies such as those described by Hacia *et al.* (1996) and Shoemaker *et al.* (1996). Briefly, these techniques involve quantitative methods for analyzing large numbers of genes rapidly and accurately. By tagging genes with oligonucleotides or using fixed probe arrays, one can employ chip technology to segregate target molecules as high density arrays and screen these molecules on the basis of hybridization. See also Pease *et al.* (1994); Fodor *et al.* (1991).

2. Treatment Protocol

Patients exhibiting neoplastic, p53-positive disease are treated with a c-Abl and optionally or alternatively, p300 polypeptide or a gene construct that comprises a c-Abl sequence, and optionally or alternatively, a p300 sequence. These genetic constructs are used in combination with a recombinant vector that comprises a nucleic acid sequence capable of expressing the desired gene sequence in the cell. The vector typically is introduced into the cell in a manner that allows expression of the encoded gene sequence at a level sufficient to effect cell function and cause an apoptotic response or to reduce cell proliferation. The following is a discussion of genetic constructs and gene transfer methods.

i. Expression Vectors

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. Thus, in certain embodiments, expression includes both transcription of a p53, p300 or c-Abl gene and translation of p53, p300 or c-Abl mRNA into the proper protein product.

In order for the construct to effect expression of a p53, p300 or c-Abl transcript, the polynucleotide encoding p53, p300 or c-Abl will be under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic

machinery of the host cell, or introduced synthetic machinery, that is required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location in relation to the polynucleotide to control RNA polymerase initiation and expression of the polynucleotide.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a *p53* or c-Abl polynucleotide is not believed to be critical, so long as it is capable of expressing the

polynucleotide in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the polynucleotide coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of the *p53* or c-Abl polynucleotide. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of polynucleotides is contemplated as well, provided that the levels of expression are sufficient to produce a growth inhibitory effect.

By employing a promoter with well-known properties, the level and pattern of expression of a polynucleotide following transfection can be optimized. For example, selection of a promoter which is active in specific cells, such as tyrosinase (melanoma), alpha-fetoprotein and albumin (liver tumors), CC10 (lung tumor) and prostate-specific antigen (prostate tumor) will permit tissue-specific expression of *p53* or c-Abl constructs. Table 1 lists several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of *p53* or c-Abl constructs. This list is not intended to be exhaustive of all the possible elements involved in the promotion of *p53* or c-Abl expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a

promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a p53, p300 or c-Abl construct. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacteriophage promoters if the appropriate bacteriophage polymerase is provided, either as part of the delivery complex or as an additional genetic expression vector.

TABLE 1

ENHANCER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ ß
ß-Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DRα
ß-Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase *I*
Metallothionein
Collagenase
Albumin Gene
α-Fetoprotein
τ-Globin
ß-Globin
c-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α_1-Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)

ENHANCER
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the *p53* construct. For example, with the polynucleotide under the control of the human PAI-1 promoter, expression is inducible by tumor necrosis factor. Table 2 illustrates several promoter/inducer combinations:

TABLE 2

Element	Inducer
MT II	Phorbol Ester (TFA) Heavy metals
MMTV	Glucocorticoids
ß-Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H_2O_2
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone

In certain embodiments of the invention, the delivery of an expression vector in a cell may be identified *in vitro* or *in vivo* by including a marker in the expression vector. The marker would result in an identifiable change to the transfected cell permitting easy identification of expression. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants. Alternatively, enzymes such as herpes simplex virus thymidine kinase (tk) (eukaryotic) or chloramphenicol acetyltransferase (CAT) (prokaryotic) may be employed. Immunologic markers also can be employed.

The selectable marker employed is not believed to be important, so long as it is capable of being expressed along with the polynucleotide encoding *p53*. Further examples of selectable markers are well known to one of skill in the art.

One will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. The inventor has employed the SV40 polyadenylation signal in that it was convenient and known to function well in the target cells employed. Also contemplated as an element of the expression construct is a terminator. These elements can serve to enhance message levels and to minimize read through from the construct into other sequences.

In certain embodiments of the invention, the cells contain nucleic acid constructs of the present invention, a cell may be identified *in vitro* or *in vivo* by including a marker in the expression construct. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable markers are well known to one of skill in the art.

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′ methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picanovirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian

message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

In preferred embodiments of the present invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis and, in some cases, integrate into the host cell chromosomes, have made them attractive candidates for gene transfer in to mammalian cells. However, because it has been demonstrated that direct uptake of naked DNA, as well as receptor-mediated uptake of DNA complexes (discussed below), expression vectors need not be viral but, instead, may be any plasmid, cosmid or phage construct that is capable of supporting expression of encoded genes in mammalian cells, such as pUC or Bluescript™ plasmid series.

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes - *gag*, *pol*, and *env* - that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the *gag* gene, termed Ψ, functions as a signal for packaging of the genome

into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a *p53* is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the *gag*, *pol* and *env* genes but without the LTR and Ψ components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a human cDNA, together with the retroviral LTR and Ψ sequences is introduced into this cell line (by calcium phosphate precipitation for example), the Ψ sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes via sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

Human adenoviruses are double-stranded DNA tumor viruses with genome sizes of approximate 36 kb (Tooza, 1981). As a model system for eukaryotic gene expression, adenoviruses have been widely studied and well characterized, which makes them an attractive system for development of adenovirus as a gene transfer system. This group of viruses is easy to grow and manipulate, and exhibit a broad host range *in vitro* and *in vivo*. In lytically infected cells, adenoviruses are capable of shutting off host protein synthesis, directing cellular machineries to synthesize large quantities of viral proteins, and producing copious amounts of virus.

The E1 region of the genome includes E1A and E1B which encode proteins responsible for transcription regulation of the viral genome, as well as a few cellular genes. E2 expression, including E2A and E2B, allows synthesis of viral replicative functions, *e.g.* DNA-binding protein, DNA polymerase, and a terminal protein that primes replication. E3 gene products prevent cytolysis by cytotoxic T cells and tumor necrosis factor and appear to be important for viral propagation. Functions associated with the E4 proteins include DNA replication, late gene expression, and host cell shutoff. The late gene products include most of the virion capsid proteins, and these are expressed only after most of the processing of a single primary transcript from the major late promoter has occurred. The major late promoter (MLP) exhibits high efficiency during the late phase of the infection (Stratford-Perricaudet and Perricaudet, 1991).

As only a small portion of the viral genome appears to be required *in cis* (Tooza, 1981), adenovirus-derived vectors offer excellent potential for the substitution of large DNA fragments when used in connection with cell lines such as 293 cells. Ad5-transformed human embryonic kidney cell lines (Graham, *et al.*, 1977) have been developed to provide the essential viral proteins *in trans*. The inventor thus reasoned that the characteristics of adenoviruses rendered them good candidates for use in targeting cancer cells *in vivo* (Grunhaus & Horwitz, 1992).

Particular advantages of an adenovirus system for delivering foreign proteins to a cell include (i) the ability to substitute relatively large pieces of viral DNA by foreign DNA; (ii) the structural stability of recombinant adenoviruses; (iii) the safety of adenoviral

administration to humans; and (iv) lack of any known association of adenoviral infection with cancer or malignancies; (v) the ability to obtain high titers of the recombinant virus; and (vi) the high infectivity of Adenovirus.

Further advantages of adenovirus vectors over retroviruses include the higher levels of gene expression. Additionally, adenovirus replication is independent of host gene replication, unlike retroviral sequences. Because adenovirus transforming genes in the E1 region can be readily deleted and still provide efficient expression vectors, oncogenic risk from adenovirus vectors is thought to be negligible (Grunhaus & Horwitz, 1992).

In general, adenovirus gene transfer systems are based upon recombinant, engineered adenovirus which is rendered replication-incompetent by deletion of a portion of its genome, such as E1, and yet still retains its competency for infection. Sequences encoding relatively large foreign proteins can be expressed when additional deletions are made in the adenovirus genome. For example, adenoviruses deleted in both E1 and E3 regions are capable of carrying up to 10 Kb of foreign DNA and can be grown to high titers in 293 cells (Stratford-Perricaudet and Perricaudet, 1991). Surprisingly persistent expression of transgenes following adenoviral infection has also been reported.

Adenovirus-mediated gene transfer has recently been investigated as a means of mediating gene transfer into eukaryotic cells and into whole animals. For example, in treating mice with the rare recessive genetic disorder ornithine transcarbamylase (OTC) deficiency, it was found that adenoviral constructs could be employed to supply the normal OTC enzyme. Unfortunately, the expression of normal levels of OTC was only achieved in 4 out of 17 instances (Stratford-Perricaudet *et al.*, 1990). Therefore, the defect was only partially corrected in most of the mice and led to no physiological or phenotypic change. These type of results therefore offer little encouragement for the use of adenoviral vectors in cancer therapy.

Attempts to use adenovirus to transfer the gene for cystic fibrosis transmembrane conductance regulator (CFTR) into the pulmonary epithelium of cotton rats have also

been partially successful, although it has not been possible to assess the biological activity of the transferred gene in the epithelium of the animals (Rosenfeld *et al.*, 1992). Again, these studies demonstrated gene transfer and expression of the CFTR protein in lung airway cells but showed no physiologic effect. In the 1991 *Science* article, Rosenfeld *et al.* showed lung expression of a1-antitrypsin protein but again showed no physiologic effect. In fact, they estimated that the levels of expression that they observed were only about 2% of the level required for protection of the lung in humans, *i.e.*, far below that necessary for a physiologic effect.

The gene for human a1-antitrypsin has been introduced into the liver of normal rats by intraportal injection, where it was expressed and resulted in the secretion of the introduced human protein into the plasma of these rats (Jaffe *et al.*, 1992). However, and disappointingly, the levels that were obtained were not high enough to be of therapeutic value.

These type of results do not demonstrate that adenovirus is able to direct the expression of sufficient protein in recombinant cells to achieve a physiologically relevant effect, and they do not, therefore, suggest a usefulness of the adenovirus system for use in connection with cancer therapy. Furthermore, prior to the present invention, it was thought that p53 could not be incorporated into a packaging cell, such as those used to prepare adenovirus, as it would be toxic. As E1B of adenovirus binds to p53, this was thought to be a further reason why adenovirus and p53 technology could not be combined.

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpes viruses may be employed. These viruses offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *in vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.* recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

2. Alternative Methods for Gene Delivery

In order to effect expression of therapeutic constructs according to the present invention, the expression vector must be delivered into a cell. As described above, one mechanism for delivery is via viral infection where the expression vector is encapsidated in an infectious virus particle.

Several non-viral methods for the transfer of expression vectors into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), polycations (Boussif *et al.*, 1995) and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

In one embodiment of the invention, the adenoviral expression vector may simply consist of naked recombinant vector. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. For example, Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding an *p53* construct may also be transferred in a similar manner *in vivo*.

Another embodiment of the invention for transferring a naked DNA expression vector into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ. DNA encoding a p53, p300 or c-Abl construct may be delivered via this method.

In a further embodiment of the invention, the expression vector may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. Liposomes form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and

dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated polynucleotide delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression vectors have been successfully employed in transfer and expression of a polynucleotide *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacteriophage promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacteriophage polymerase.

Another mechanism for transferring expression vectors into cells is receptor-mediated delivery. This approach takes advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993). Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1993). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales

et al., 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that an adenoviral expression vector also may be specifically delivered into a cell type such as lung, epithelial or tumor cells, by any number of receptor-ligand systems, with or without liposomes. For example, epidermal growth factor (EGF) may be used as the receptor for mediated delivery of p53, p300 or cAbl construct in many tumor cells that exhibit upregulation of EGF receptor. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties.

In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene therapy refers to the isolation of cells from an animal, the delivery of a polynucleotide into the cells, *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues. Anderson *et al.*, U.S. Patent 5,399,346, and incorporated herein in its entirety, disclose *ex vivo* therapeutic methods. During *ex vivo* culture, the expression vector can express the p53 construct. Finally, the cells may be reintroduced into the original animal, or administered into a distinct animal, in a pharmaceutically acceptable form by any of the means described below.

D. Treatment of p53-Negative Cancers using p53 and c-Abl

A patient presenting a tumor that does not express wild-type p53 will be treated with a combination of p53 and c-Abl and/or p300. The patient will be treated with a pharmaceutically acceptable form of the c-Abl and/or p300 genes or gene products, as described above, and in addition, will be treated with a p53 gene or gene product. Again, a typical treatment regimen will involve repeated administrations over a six week period. During

this time, the tumor will be monitored for absence of tumor progression, response or toxicity and the doses adjusted accordingly.

E. Combination Therapy

Tumor cell resistance to DNA damaging agents represents a major problem in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy by combining it with alternative therapies. For example, the herpes simplex thymidine kinase gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, *et al.*, 1992). In the context of the present invention, it is contemplated that c-Abl/p300 therapy could be used similarly in conjunction with chemo- or radiotherapeutic intervention. It also may prove effective to combine c-Abl/p300 therapy with immunotherapy.

To kill tumor cells or reduce their proliferation, using the methods and compositions of the present invention, one would generally contact a "target" cell with (i) radiation or a chemotherapeutic and (ii) a therapeutic protein or gene. These compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with c-Abl, p300 and p53 protein or gene, at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes c-Abl protein or gene and/or p300, and the other includes the p53 protein or gene.

Alternatively, the protein or gene therapy treatment may precede or follow the standard chemo- or radiotherapy treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and an expression construct are applied separately to the cell, one generally would ensure that a significant period of time did not expire between the time of each delivery, such that the agent and expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both modalities within about 12-24 hours of each other and, more

preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either c-Abl or p300 genes or proteins, and optionally p53 genes or proteins, with the other agent will be desired. Various combinations may be employed, where c-Abl, and/or p300, and optionally p53 will be "A" and the other agent is "B", as exemplified below:

A/B/A B/A/B B/B/A A/A/B B/A/A A/B/B B/B/B/A B/B/A/B

A/A/B/B A/B/A/B A/B/B/A B/B/A/A B/A/B/A B/A/A/B B/B/B/A

A/A/A/B B/A/A/A A/B/A/A A/A/B/A A/B/B/B B/A/B/B B/B/A/B

Other combinations are contemplated. Again, to achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the cell.

Agents or factors suitable for use in a combined therapy are any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include radiation and waves that induce DNA damage such as, γ-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as "chemotherapeutic agents," function to induce DNA damage, all of which are intended to be of use in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated to be of use, include, *e.g.*, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP) and even hydrogen peroxide. The invention also encompasses the use of a combination of one or more DNA damaging agents, whether radiation-based or actual compounds, such as the use of X-rays with cisplatin or the use of cisplatin with etoposide. In certain embodiments, the use of

cisplatin in combination with a c-Abl or p300 expression construct is particularly preferred as this compound.

In treating cancer according to the invention, one would contact the tumor cells with an agent in addition to the expression construct. This may be achieved by irradiating the localized tumor site with radiation such as X-rays, UV-light, γ-rays or even microwaves. Alternatively, the tumor cells may be contacted with the agent by administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a compound such as, adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, or more preferably, cisplatin. The agent may be prepared and used as a combined therapeutic composition, or kit, by combining it with an expression construct, as described above.

Agents that directly cross-link nucleic acids, specifically DNA, are envisaged to facilitate DNA damage leading to a synergistic, antineoplastic combination with c-Abl and/or p300. Agents such as cisplatin, and other DNA alkylating agents may be used. Cisplatin has been widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m^2 for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intratumorally or intraperitoneally.

Agents that damage DNA also include compounds that interfere with DNA replication, mitosis and chromosomal segregation. Such chemotherapeutic compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in a clinical setting for the treatment of neoplasms, these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m^2 at 21 day intervals for adriamycin, to 35-50 mg/m^2 for etoposide intravenously or double the intravenous dose orally.

Agents that disrupt the synthesis and fidelity of nucleic acid precursors and subunits also lead to DNA damage. As such a number of nucleic acid precursors have been developed. Particularly useful are agents that have undergone extensive testing and are readily available.

As such, agents such as 5-fluorouracil (5-FU), are preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage DNA, on the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

The inventors propose that the regional delivery of expression constructs to patients will be a very efficient method for delivering a therapeutically effective gene to counteract the clinical disease. Similarly, the chemo- or radiotherapy may be directed to a particular, affected region of the subjects body. Alternatively, systemic delivery of expression construct and/or the agent may be appropriate in certain circumstances, for example, where extensive metastasis has occurred.

F. Pharmaceutical Compositions and Routes of Administration

Aqueous compositions of the present invention will have an effective amount of a compound that increases the expression of wild-type p53, for example p300 or c-Abl gene products or p300 or c-Abl gene constructs. Such compositions will generally be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Also included in various embodiments of the present invention are standard chemotherapeutics in their various pharmaceutical forms.

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients, its use in the therapeutic compositions is contemplated. Supplementary active ingredients, such as other anti-cancer agents, can also be incorporated into the compositions.

In addition to the compounds formulated for parenteral administration, such as those for intravenous or intramuscular injection, other pharmaceutically acceptable forms include, *e.g.*, tablets or other solids for oral administration; time release capsules; and any other form currently used, including cremes, lotions, mouthwashes, inhalants and the like.

The active compounds of the present invention will often be formulated for parenteral administration, *e.g.*, formulated for injection *via* the intravenous, intramuscular, sub-cutaneous, or even intraperitoneal routes. The preparation of an aqueous composition that contains a compound or compounds that increase the expression of wild-type p53 will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the

addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

In certain aspects of the invention, compounds may be injected directly into a tumor. In other aspects, the route of delivery is by a slow drip into the circulatory system of the patient.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The active compounds may be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene

glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

In certain cases, the therapeutic formulations of the invention could also be prepared in forms suitable for topical administration, such as in cremes and lotions. These forms may be used for treating skin-associated diseases, such as various sarcomas.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, with even drug release capsules and the like being employable.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for

intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 mL of isotonic NaCl solution and either added to 1000 mL of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

G. Kits

All the essential materials and reagents required for determining wild-type p53 in a sample or for increasing the expression of wild-type p53 using c-Abl and p300 in tumor cells may be assembled together in a kit. When the components of the kit are provided in one or more liquid solutions, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being particularly preferred.

For the detection of wild-type p53, the kit may contain materials for PCR analyses, such primers, buffers and appropriate solvents. Alternatively, if the detection is via immunologic means, the kit may contain antibodies directed to the p53, secondary antibodies that binding primary antibodies, labels or signal generating compounds (either conjugated or unconjugated) and various reagents for the generation and detection of signals.

For *in vivo* use, an inducer of wild-type p53 expression, alone or in combination with, a expression vectors may be formulated into a single or separate pharmaceutically acceptable syringeable composition. In this case, the container means may itself be an inhalant, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the body, such as the lungs, injected into an animal, or even applied to and mixed with the other components of the kit.

The components of these kits may also be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means. The kits of the invention may also include an instruction sheet defining administration of wild-type p53 and c-Abl gene therapy agents, or explaining the assays for determining p53 levels in samples.

The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials are retained. Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of an animal. Such an instrument may be an inhalant, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle. Other instrumentation includes devices that permit the reading or monitoring of reactions *in vitro*.

H. Examples

The following examples are included to demonstrate preferred embodiments of the present invention. It should be appreciated by those of skill in the art that that techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar results without departing from the spirit and scope of the invention.

Example 1: Role for c-Abl in Growth Arrest Response to DNA Damage

METHODS. Transient transfections were done with lipofectamine (GIBCO-BRL). Cells were collected 48 h after transfection. Luciferase was assayed with an

enhanced luciferase assay kit (1800K, Analytical Luminescent). Histone HI kinase was assayed as described.

Human MCF-7 breast cancer cells ($p53^{+/+}$) were treated with 5 Gy IR at room temperature with a Gammacell 1000 (Atomic Energy of Canada) with a ^{187}Cs source emitting at a fixed dose rate of 0.76 Gy min. Lysates were prepared in 0.5% NP-40 lysis buffer as described. MCF-7/pSR and MCF-7/c-Abl (K-R) cells were blocked at G1/S phase by a 12 h treatment with 10 μM aphidicolin. Cells were then washed, irradiated and fed with fresh medium containing 10% FCS.

Asynchronous populations of *abl*$^{+/+}$ and *abl*$^{-/-}$ MEFs (Tybulewicz *et al.*, 1991) were exposed to IR and the percentage of cells in S phase assessed at 20 h. BrdU was added 30 min before collection. Cells were stained for DNA content with propidium and for DNA synthesis with a fluorescein conjugated anti-BrdU antibody (Boehringer Mannheim).

RESULTS: The product of the c-Abl gene is a non-receptor tyrosine kinase that is localized to the nucleus and cytoplasm. Ionizing radiation (IR) activates c-Abl. Similar results were obtained with the alkylating agents cisplatinum and mitomycin C. Cells deficient in c-Abl fail to activate Jun kinase (JNK/SAP kinase) following IR or alkylating agent exposure and that reconstitution of c-Abl in these cells restores that response. In contrast, the stress response to tumor necrosis factor is stimulated by a c-Abl-independent mechanism. These findings indicate that c-Abl is involved in the stress response to DNA-damaging agents.

In addition to sharing structural features with members of the src family, c-Abl contains actin binding and DNA binding domains. The finding that c-Abl associates with the retinoblastoma (Rb) protein has suggested a potential role for c-Abl in regulating the cell cycle (Welch and Wang, 1993). Other studies have shown that overexpression of c-Abl induces an arrest in G_1 phase (Sawyers *et al.*, 1994; Mattioni *et al.*, 1995). Phosphorylation of c-Abl on multiple sites by $p34^{cdc2}$ during mitosis has also supported a role in G2 phase (Kipreos and Wany, 1990). The phosphorylation of c-Abl in mitotic cells inhibits DNA binding (Kipreos

and Wang, 1992). While these findings have suggested that c-Abl contributes to regulation of the cell cycle, the present studies demonstrate that c-Abl is activated by DNA-damaging agents. DNA damage could dissociate c-Abl from a complex with other proteins and thereby contribute to interactions with potential substrates. In this context, binding of c-Abl to the first Crk SH3 domain targets phosphorylation of c-Crk on Tyr221 (Feller *et al.*, 1994; Ren *et al.*, 1994; Feller *et al.*, 1994). Since DNA damage is associated with arrest of cells in G_1 and G_2 phases, c-Abl activation could play a role in regulating these responses to genotoxic stress. Alternatively, while overexpression of c-Abl arrests cells in G_1 phase, activation of c-Abl by DNA damage may regulate distinct stress pathways that include SAP kinase.

To determine whether c-Abl affects the function of p53, MCF-7 cells were co-transfected with a construct containing the luciferase gene driven by a p53 enhancer from the *MDM2* promoter (Barak *et al.*, 1994), and vectors expressing wild-type c-Abl, a kinase-inactive K(290)R mutant (Sawyers *et al.*, 1994), or a kinase-active mutant, designated ΔPro^4 which is deleted at the p53-binding domain (Goga *et al.*, 1995). Co-transfections of the reporter with wild-type or kinase-inactive c-Abl (K-R), but not c-AblΔPro^4 resulted in induction of luciferase activity (FIG. 1A). By contrast, c-Abl expression had no detectable effect on activation of an SV40-luciferase construct (FIG. 1a). As transcription of p21 is regulated by p53 (ref. 6), the transfection of cells with c-Abl to induce p21 was investigated. It was found that p21 expression increased in the c-Abl and c-Abl (K-R), but not the c-AblΔPro^4 transfectants (FIG. 1B). Despite induction of p21; which is an inhibitor of Cdk2 (refs 7-10), by both wild-type c-Abl and c-Abl (K-R), Cdk2 was downregulated only in cells transfected with wild-type c-Abl (FIG. 1C), which correlates with the ability of wild-type c-Abl, but not c-Abl (K-R) or c-AblΔPro^4 to inhibit growth in fibroblasts and MCF-7 cells. These findings suggest that the kinase activity and p53-binding domain of c-Abl are involved in downregulation of Cdk2 and thus in growth arrest, and that such effects are not exclusively mediated by activation of p21.

To determine the involvement of c-Abl in DNA-damage-dependent growth arrest MCF-7 cells were prepared to stably express the dominant-negative c-Abl $(K\text{-}R)^2$, which

effectively inhibits the increase in c-Abl kinase activity induced by ionizing radiation control cells (FIG. 2A). Irradiation of MCF-7/pSR or MCF-7/c-Abl (K-R) cells caused an increase in p53, p21 and GADD45, but not in c-Myc (which is not dependent on p53) (FIG. 2B); there was also increased binding of p21 to Cdk2 in MCF-7/pSR and in MCF-7/c-Abl (K-R) cells (FIG. 2C). Although Cdk2 was downregulated in irradiated MCF-7/pSR cells, there was little effect on Cdk2 activity in MCF-7/c-Abl (K-R) cells (FIG. 2D). These results indicate that c-Abl kinase is not required for transactivation of p21 and GADD45 by p53 in response to DNA damage, but is required for Cdk2 downregulation.

The role of c-Abl in growth arrest induced by ionizing radiation was tested by the ability of irradiated MCF-7/pSR and MCF-7/c-Abl (K-R) cells to reduce the number of cells in S phase. Using bromo-deoxyuridine (BrdU) labeling and bivariate fluorescence-activated cell-sorting (FACS) analysis, it was found that more irradiated MCF-7/pSR cells arrested in G1 compared with controls (FIG. 3A). Only 15% of cells were in S phase 24h after receiving 5 Gy radiation compared with untreated cells. However, irradiated MCF-7/c-Abl (K-R) cells were less affected, with the S-phase population being 45% of untreated samples (FIG. 3A). Similar results were obtained with two independently isolated MCF-7/c-Abl (K-R) clones (FIG. 3B). To confirm the link between c-Abl and radiation induced growth arrest, mouse embryo fibroblasts (MEFs) deficient in c-Abl (*abl* $^{-/-}$ mice with targeted disruption of the c-*abl* gene) were studied (FIG. 3C). Wild-type MEFs after 5 Gy radiation had an S-phase population that was 45% of that for untreated cells (FIG. 3D). By contrast, irradiated *abl*$^{-/-}$ MEFs had more than 70% of cells in S phase compared with controls (FIG. 3D). Exposure of the wild-type and *abl*$^{-/-}$ MEFs to 20 Gy radiation partially inhibited arrest in G1 in the *abl*$^{-/-}$ cells, indicating that the c-Abl kinase function is necessary for radiation-induced G1 arrest.

As c-Abl and p53 bind together *in vitro*, the inventors determined whether they were associated in irradiated cells. No p53 was detected in anti-c-Abl immunoprecipitates from MCF-7/pSR cells that had not been irradiated (FIG. 4A), but binding of c-Abl and p53 was evident at 30 min and maximal at 3-5h after irradiation (FIG. 4A). The small

amount of constitutive binding of c-Abl to p53 found in MCF-7/c-Abl (K-R) cells was stimulated by irradiation (FIG. 4A).

To investigate further the role of p53 in growth regulation by c-Abl, *p53*$^{-/-}$ fibroblasts were transfected with wild-type c-Abl and c-Abl (K-R). Analysis of p21 levels and Cdk2 activity failed to reveal any effect with either vector (FIG. 4B). However, Cdk2 was downregulated in *p53*$^{+/+}$ fibroblasts after transfection with wild-type c-Abl, but no c-Abl (K-R) (FIG. 4B). These results show that c-Abl kinase inhibits Cdk2 by a p53-dependent mechanism. The inventors found that the effect on Cdk2 activity of irradiating *abl*$^{-/-}$ cells did not decrease as much as in wild-type MEFs (FIG. 4C). In *p21*$^{-/-}$ fibroblasts, it was found that p21 only partially mediates the action of p53 in arresting radiation-damaged cells in G1 (refs 12, 13). In support of previous findings, expression of c-Abl (K-R) had no effect on Cdk2 activity in *p21*$^{-/-}$ or *p21*$^{+/+}$ cells (FIG. 4D); whereas transfection of wild-type c-Abl into *p21*$^{-/-}$ cells inhibited Cdk2 (FIG. 4D) and growth, indicating that these effects of c-Abl depend on p53 but not p21. It is possible, however, that growth arrest following transfection with c-Abl inhibits Cdk2 activity.

These results, obtained using a dominant-negative c-Abl mutant, suggest that c-Abl regulates growth in response to genotoxic stress. Because c-Abl associates with the retinoblastoma protein Rb and overexpression of kinase-defective c-Abl abrogates Rb induced growth arrest, mutant c-Abl may prevent G1 arrest in response to DNA damage by inactivating Rb. But as irradiated cells expressing c-Abl (K-R) do not arrest in G1 because Cdk2 is not inhibited, the effect probably occurs upstream of Rb. The present inventors have demonstrated that c-Abl functions in the cellular response to DNA damage through p53-dependent pathways, confirmed by the failure of the c-AblΔPro[4] mutant to bind p53 and downregulate Cdk2 and arrest growth. It has also been shown that the kinase-defective c-Abl (K-R) mutant induces p53 transactivation but no G1 arrest: although c-Abl stimulates the transcriptional activity of p53, as measured by expression of its target gene *p21*, a different mechanism is used by p21 in growth arrest because c-Abl mediated transactivation is kinase-independent and Cdk2 down-regulation

and growth arrest by the c-Abl kinase are p21-independent. These results support a novel c-Abl/p53 dependent, but p21-independent, mechanism for the regulation of Cdk2.

Example 2: Genotoxic Drugs Induce Interaction of c-Abl and p53

METHODS: *Cell Culture* - MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% heat-inactivated fetal bovine serum (Sigma), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Null pSRαMSVtKneo, pSRαMSVc-AblK(290)-RtKNeo (Sawyers *et al.*, 1994), or E6Neo (Scheffner *et al.*, 1990) vectors were stably introduced into cells by LipofectAMINE (Life Technologies, Inc.) and selection in G418. The cells were treated with 10 μM ara-C (Sigma) or 10 μg/ml MMS (Sigma). Cell cycle analysis was performed as described (Yuan *et al.*, 1996).

Immunoprecipitations and Immunoblot Analysis - Cell lysates were prepared as described (Yuan *et al.*, 1996) and incubated with rabbit anti-Cdk2 (sc-163, Santa Cruz Biotechnology, San Diego, CA), mouse anti-c-Abl (Ab-3, Oncogene Science, Cambridge, MA), mouse anti-p53 (Ab-6, Oncogene Science) antibodies for 6-12 h at 4°C and then for 60 min with protein A-Sepharose. For mouse antibodies, 10 μg/sample of rabbit anti-mouse IgG was added and incubated for 60 min before the addition of protein A beads. The immune complexes were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters and analyzed by immunoblotting using an ECL (Amersham Corp.) detection system.

Kinase Assays - Cdk2 kinase assays were performed as described (Yuan *et al.*, 1996). For c-Abl kinase assays, recombinant SHPTP1 protein (100 μg/ml) was used as substrate (Kharbanda *et al.*, 1996), and the reaction was incubated for 30 min at 30°C.

RESULTS: Treatment of NIH3T3 cells with Ara-C is associated with activation of the c-Abl kinase (Kharbanda *et al.*, 1995b). To determine whether the activation of c-Abl by genotoxic drugs is associated with growth arrest, the inventors prepared MCF-7

cells that stably express a dominant negative, kinase-inactive c-Abl (K290R) mutant (Sawyers *et al.*, 1994). Treatment of control MCF-7/pSR cells with ara-C was associated with increases in c-Abl kinase activity. By contrast, there was no detectable c-Abl activity in untreated or ara-C-treated MCR-7/c-Abl(K-R) cells.

Other studies have demonstrated that c-Abl binds to p53 *in vitro* (Goga *et al.*, 1996). Consequently, it was asked whether ara-C induces an interaction between c-Abl and p53 *in vivo*. As a control, the inventors also prepared cells that express the human papillomavirus E6 protein to promote degradation of p53 (Scheffner *et al.*, 1990). The MCF-7/E6 cells responded to ara-C with induction of c-Abl activity.

In coimmunoprecipitation studies, there was no detectable p53 in the c-Abl immunoprecipitates from control MCF-7/pSR cells. However, binding of c-Abl and p53 was detectable when MCF-7/pSR cells were treated with ara-C for 1 h. The association of c-Abl and p53 was also increased by treatment of MCF-7/c-Abl(K-R), but not MCF-7/E6, cells with ara-C. These findings indicated that ara-C induces a c-Abl-p53 interaction independent of the c-Abl kinase function.

Treatment of MCR-7/pSR and MCF-7/c-Abl(K-R) cells with ara-C was also associated with increases in p53 expression, while there was no apparent effect of this agent on p53 levels in MCF-7/E6 cells. As transcription of p21 is regulated by p53 (El-Deiry *et al.*, 1993), the MCF-7/pSR and MCF-7/Abl(K-R), but not the MCR-7/E6, cells responded to ara-C with induction of p21 expression. p21 is an inhibitor of Cdk2 (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1995). However, while p21 was induced in both MCF-7/pSR and MCF-7/c-Abl(K-R) cells and was bound to Cdk2 in both cell types, Cdk2 activity was down-regulated by ara-C treatment in only the MCF-7/pSR cells.

As observed with ara-C, MMS exposure resulted in increased c-Abl activity in the MCF-7/pSR and MCF-7/E6 cells, but not the MCF-7/c-Abl(K-R) cells. MMS increased binding of c-Abl and p53 in MCF-7/pSR and MCF-7/c-Abl(K-R), but not MCF-7/E6, cells. MMS also induced the expression of p53 and p21 in only the MCF-7/pSR and

MCF-7/c-Abl(K-R) cells. The MMS-induced increases in p21 levels were associated with binding of p21 to Cdk2. However, while Cdk2 activity was down-regulated in MMS-treated MCF-7/pSR cells, there was little effect of MMS on Cdk2 activity in the MCF-7/c-Abl(K-R) cells.

The role of c-Abl in genotoxic drug-induced growth arrest was assessed by the ability of MMS to decrease populations of S phase cells. The number of MCF-7/pSR cells in S phase at 9 h after MMS treatment was 67% of that obtained for untreated cells (Table I). By contrast, MMS-treated MCF-7/c-Abl(K-R) cells exhibited partial inhibition of the G1 response with an S phase population after treatment of over 80% of that for untreated cells (Table I). Similar findings were obtained with two independently isolated MCF-7/c-Abl(K-R) clones, while the G_1 arrest response was almost completely abrogated in the MCF-7/E6 cells (Table I). These findings suggest that, as found for the down-regulation of Cdk2, c-Abl kinase activity is involved in MMS-induced G1 arrest by a p53-dependent mechanism.

TABLE I

MMS Induces p53-Mediated G_1 Arrest by c-Abl Dependent Mechanisms

	S phase cells		
Transfectant	Control	Treated	Treated Control G
MCF-7/pSR	38.8 = 3.2	26.2 = 2.8	67.5
MCF-7/c-Abl(K-R)			
Clone a	38.4 = 3.9	32.2 = 4.6	83.9
Clone b	37.9 = 3.0	30.6 = 2.8	80.7
MCF-7/E6	36.6 = 4.8	34.5 = 3.1	94.3

To substantiate the link between c-Abl and DNA damage-induced growth arrest, the effects of MMS on mouse embryo fibroblasts (MEFs) deficient in c-Abl ($Abl^{-/-}$) (Tybulewicz *et al.*, 1991) were studied. Wild type MEFs responded to MMS with induction of the c-Abl kinase, while there was no detectable c-Abl activity or protein in MMS-treated $Abl^{-/-}$ cells. Both wild type and $Abl^{-/-}$ MEFs responded to MMS with increases in p53 and p21 levels. These findings indicated that c-Abl is not necessary for DNA damage-induced increases in the transactivation function of p53. However, despite

induction of p21 in both cell types, Cdk2 activity was significantly down-regulated in MMS-treated wild-type, but not $Abl^{-/-}$, MEFs. Wild-type MEFs also responded to MMS with G1 arrest. The number of wild type MEFs in S phase at 9 h after MMS treatment was 51% of that obtained for untreated cells (FIG. 5). By contrast, MMS-treated $Abl^{-/-}$ cells had an S phase population which was 71% of that for untreated cells (FIG. 5). These findings suggest that c-Abl is involved in the down-regulation of Cdk2 and that the G1 arrest response involves mechanisms other than activation of p21.

The present results demonstrate that treatment of cells with ara-C or MMS is associated with binding of c-Abl and p53. Similar findings have been obtained with the genotoxic topoisomerase inhibitors camptothecin and etoposide and with ionizing radiation exposure (Yuan *et al.*, 1996). Thus, diverse types of DNA damage activate c-Abl (Kharbanda *et al.*, 1996b; Kharbanda *et al.*, 1995), and the interaction of c-Abl with p53. However, the finding that c-Abl binds to p53 following treatment of the MCF-7/c-Abl(K-R) cells with genotoxic agents indicates that activation of the c-Abl kinase is not necessary for the interaction. Moreover, the demonstration that expression of p53 and p21 is increased in MMS-treated MCF-7/c-Abl(K-R) and $Abl^{-/-}$ cells suggests that c-Abl is not necessary for inducing certain transactivation functions of p53 following genotoxic stress. Conversely, the findings that c-Abl is activated by DNA-damaging agents in MCF-7/E6 and $p53^{-/-}$ cells indicates that p53 is dispensable for c-Abl activation.

The present results also indicate that activation of the c-Abl kinase is necessary for the complete G_1 arrest response to genotoxic drugs. Induction of p21 expression and binding of this Cdk inhibitor to Cdk2 by genotoxic stress is insufficient for the complete down-regulation of Cdk2. Rather, findings indicate that induction of c-Abl kinase activity is also involved in the down-regulation of Cdk2 and growth arrest. Recent work has shown that overexpression of kinase active, but not inactive, c-Abl in $p21^{-/-}$ fibroblasts is associated with down-regulation of Cdk2 and growth arrest (Yuan *et al.*, 1996). In concert with these results is the demonstration that p53-dependent events can inhibit cell growth through p21-independent mechanisms (Deng *et al.*, 1995; Hirano *et al.*, 1995; Brugarolas *et al.*, 1995). Taken together, these findings indicate that genotoxic agents

induce the down-regulation of Cdk2 and G1 arrest by a mechanism dependent on activation of c-Abl kinase and binding of c-Abl to p53, but independent of p21.

* * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention.

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CLAIMS

1. A method for increasing p53-mediated apoptosis in a tumor cell comprising the step of increasing the activity level of at least one of c-Abl and p300 in said tumor cell.

2. The method of claim 1, wherein said tumor cell is derived from a tissue selected from the group consisting of brain, lung, liver, spleen, kidney, lymph node, small intestine, blood cells, pancreas, colon, stomach, breast, endometrium, prostate, testicle, ovary, skin, head and neck, esophagus, bone marrow and blood tissue..

3. The method of claim 1, wherein the activity levels of both c-Abl and p300 are increased in said tumor cell.

4. The method of claim 1, wherein the activity level is increased by providing at least one of a c-Abl polypeptide and a p300 polypeptide to said tumor cell in an amount effective to increase p53-mediated apoptosis.

5. The method of claim 1, wherein the activity level is increased by providing at least one of a nucleic acid encoding c-Abl and a nucleic acid encoding p300 to said tumor cell, wherein said nucleic acid is operably linked to a promoter active in eukaryotic cells in an amount effective to increase p53-mediated apoptosis.

6. The method of claim 1, wherein the activity level is increased by providing an agent to said cell that increases the expression or stability of at least one of c-Abl and p300 in an amount effective to increase p53-mediated apoptosis.

7. The method of claim 4, wherein said polypeptide is delivered in a liposome.

8. The method of claim 5, wherein said nucleic acid is contained in an expression vector.

9. The method of claim 8, wherein said expression vector is a viral vector.

10. The method of claim 9, wherein said viral vector is selected from the group consisting of herpesvirus, adenovirus, vaccinia virus, retrovirus and adeno-associated virus.

11. The method of claim 10, wherein said nucleic acid is encapsulated in a viral particle.

12. The method of claim 1, wherein said tumor cell is defective in p53, and said method further comprises providing to said tumor cell a nucleic acid encoding a wild-type p53 operably linked to a promoter active in eukaryotic cells.

13. The method of claim 12, wherein said nucleic acid is contained in an expression vector

14. The method of claim 13, wherein said expression vector is a viral vector.

15. The method of claim 14, wherein said viral vector is selected from the group consisting of herpesvirus, adenovirus, vaccinia virus, retrovirus and adeno-associated virus.

16. The method of claim 1, further comprising contacting said cell with ionizing radiation.

17. The method of claim 1, further comprising contacting said cell with a chemotherapeutic agent.

18. The method of claim 13, wherein said ionizing radiation is x-irradiation or γ-irradiation.

19. The method of claim 14, wherein said chemotherapeutic agent is selected from the group consisting of mitomycin C, etoposide, genistin, cisplatin, 5-FU, adriamycin, doxorubicin, actinomycin D, verapamil, nitrosourea, ara-C and camptothecin.

20. A method for treating a patient having a tumor comprising the step of increasing the activity level of at least one of c-Abl and p300 in cells of said tumor.

21. The method of claim 20, wherein the activity level is increased by providing at least one of a c-Abl polypeptide and a p300 polypeptide to said tumor in an amount effective to increase p53-mediated apoptosis in cells thereof.

22. The method of claim 20 wherein the activity level is increased by providing at least one of a nucleic acid encoding c-Abl and a nucleic acid encoding p300 to said tumor, wherein said nucleic acid is operably linked to a promoter active in eukaryotic cells in an amount effective to increase p53-mediated apoptosis in cells thereof.

23. The method of claim 20, wherein the activity level is increased by providing an agent to said tumor that increases the expression or stability of at least one of c-Abl and p300 in an amount effective to increase p53-mediated apoptosis in cells thereof.

24. The method of claim 21, wherein said polypeptide is provided via intratumoral injection

25. The method of claim 22, wherein said nucleic acid is provided via intratumoral injection.

26. The method of claim 23, wherein said agent is provided via intratumoral injection.

27. The method of claim 20, wherein said tumor comprises cells which lack p53 activity, and said method further comprises providing to said tumor cell a nucleic acid encoding a wild-type p53 operably linked to a promoter active in eukaryotic cells.

28. The method of claim 20, further comprising contacting said cell with ionizing radiation.

29. The method of claim 20, further comprising contacting said cell with a chemotherapeutic agent.

30. A method of screening a candidate substance for p53-stimulatory activity comprising the steps of:

(i) providing a eukaryotic cell expressing a functional p300 polypeptide;
(ii) contacting said cell with said candidate substance; and
(iii) determining the effect of said candidate substance on the p300 level of said cell,

wherein an increase in the p300 level in said cell, as compared to an untreated cell, indicates that said candidate substance increases p53 activity.

31. The method of claim 30, wherein said p300 level is measured by Western blot or ELISA.

32. The method of claim 30, wherein said candidate substance is a nucleic acid encoding a polypeptide operably linked to a promoter active in eukaryotic cells and said contacting comprises transferring said nucleic acid into said cell.

33. The method of claim 32, wherein said transferring is achieved by transfection, lipofection, protoplast fusion, bombardment, electroporation or viral infection.

34. A method of screening a candidate substance for p53-stimulatory activity comprising the steps of:

(i) providing a eukaryotic cell expressing a functional p300 polypeptide;

(ii) contacting said cell with said candidate substance; and

(iii) determining the effect of said candidate substance on the p300 level of said cell,

wherein an increase in the p300 level in said cell, as compared to an untreated cell, indicates that said candidate substance increases p53 activity.

35. The method of claim 34, wherein said c-Abl level is measured by Western blot or ELISA.

36. The method of claim 34, wherein said candidate substance is a nucleic acid encoding a polypeptide operably linked to a promoter active in eukaryotic cells and said contacting comprises transferring said nucleic acid into said cell.

37. The method of claim 36, wherein said transferring is achieved by transfection, lipofection, protoplast fusion, bombardment, electroporation or viral infection.

38. Use of at least one of a p300 and a c-Abl polypeptide for the preparation of a pharmaceutical composition effective to increase p53-mediated apoptosis in a cell.

39. Use of at least one of a nucleic acid encoding a p300 polypeptide and a nucleic acid encoding a c-Abl polypeptide for the preparation of a pharmaceutical composition effective to increase apoptosis in a cell.

40. Use of a p300 polypeptide or gene coding therefor for the preparation of a pharmaceutical composition for the treatment of cancer.

41. Use of a p300 polypeptide or gene coding therefor for the preparation of a pharmaceutical composition for the treatment of cancer.

FIG. 1A

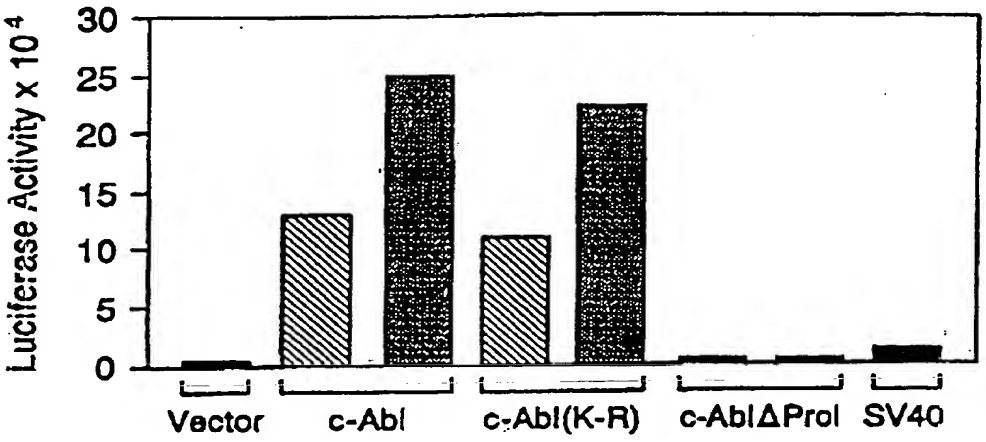


FIG. 1B

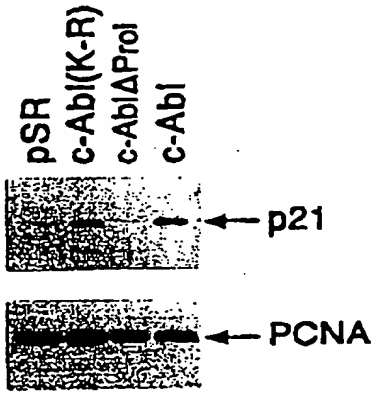


FIG. 1C

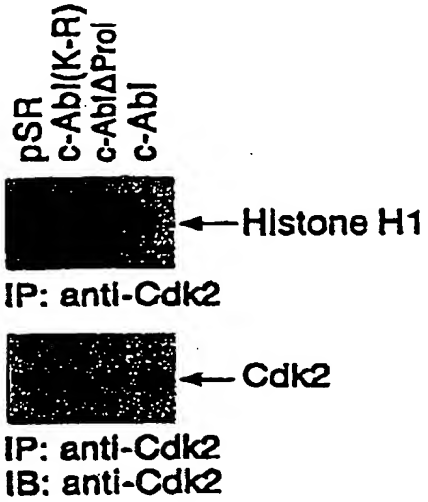
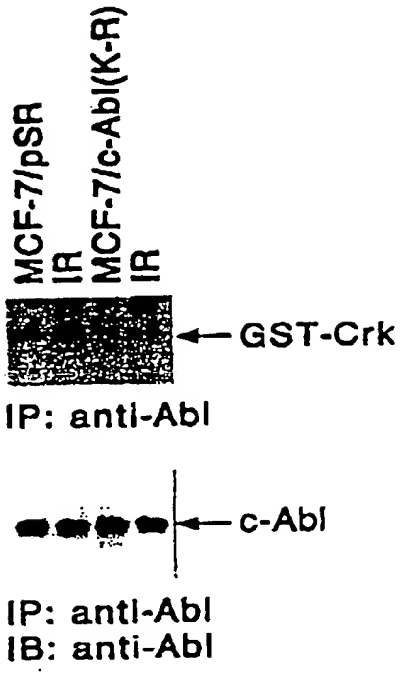


FIG. 2A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IR
MCF-7/c-Abl(K-R)
IR
GST-Crk
IP: anti-Abl
c-Abl
IP: anti-Abl
IB: anti-Abl

FIG. 2B

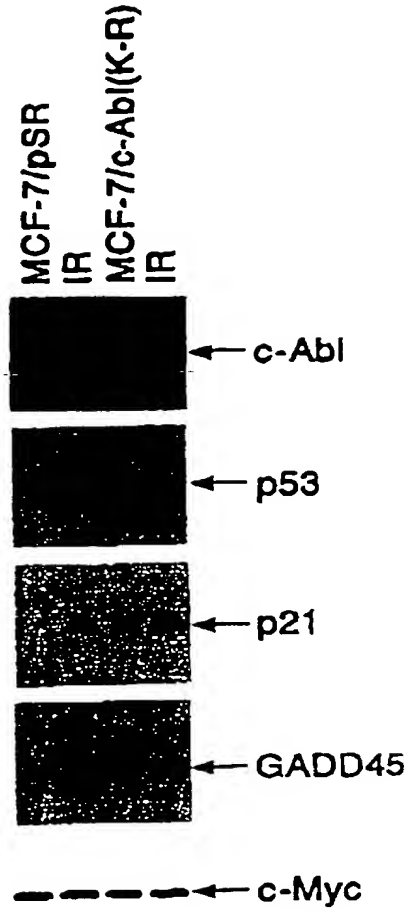

MCF-7/pSR
IR
MCF-7/c-Abl(K-R)
IR
c-Abl
p53
p21
GADD45
c-Myc

FIG. 2C

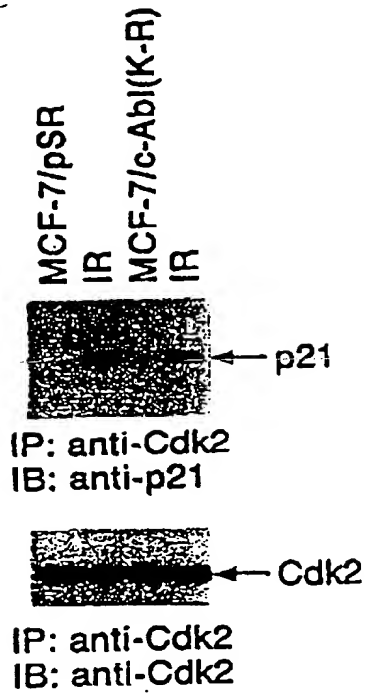
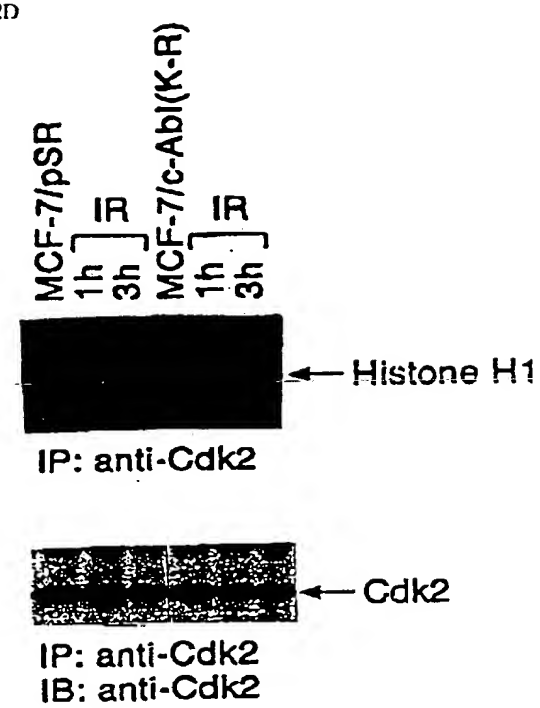

MCF-7/pSR
IR
MCF-7/c-Abl(K-R)
IR
p21
IP: anti-Cdk2
IB: anti-p21
Cdk2
IP: anti-Cdk2
IB: anti-Cdk2

FIG. 2D


MCF-7/pSR
IR
1h
3h
MCF-7/c-Abl(K-R)
IR
1h
3h
Histone H1
IP: anti-Cdk2
Cdk2
IP: anti-Cdk2
IB: anti-Cdk2

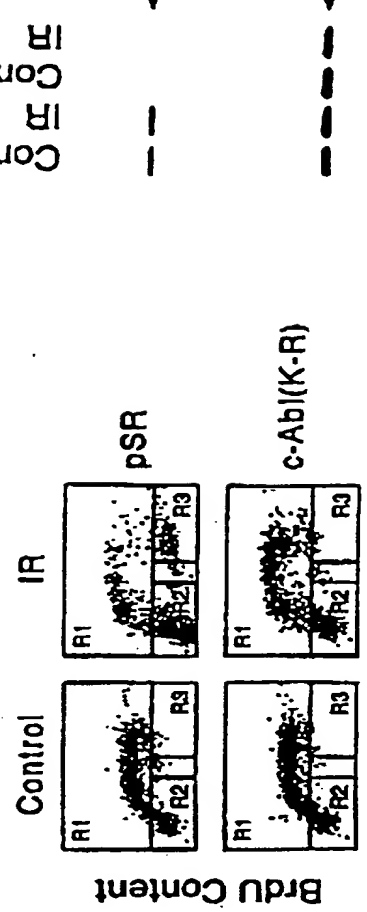


FIG. 3A

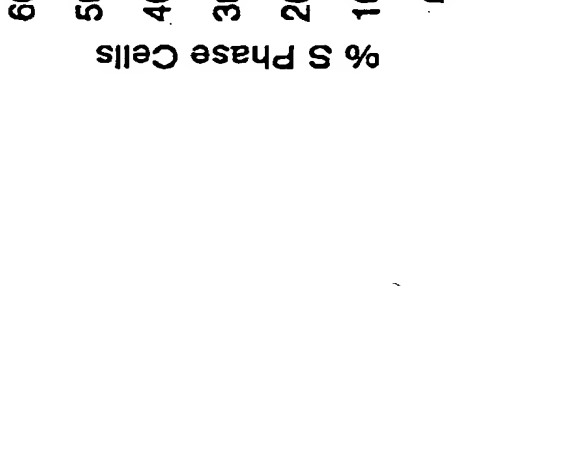


FIG. 3B

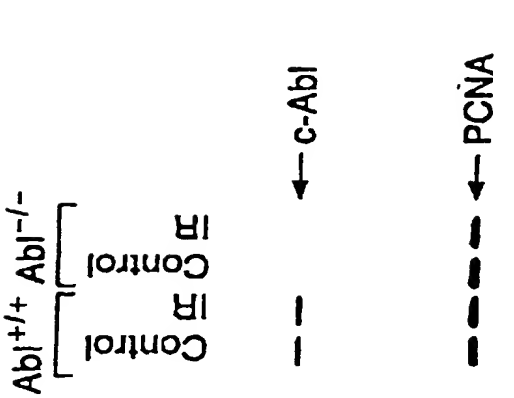


FIG. 3C

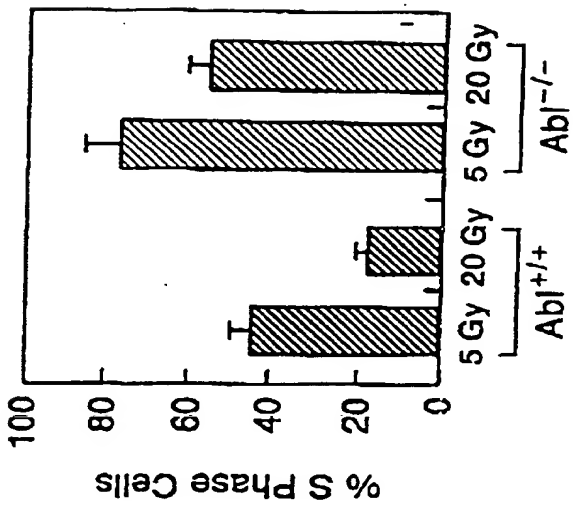
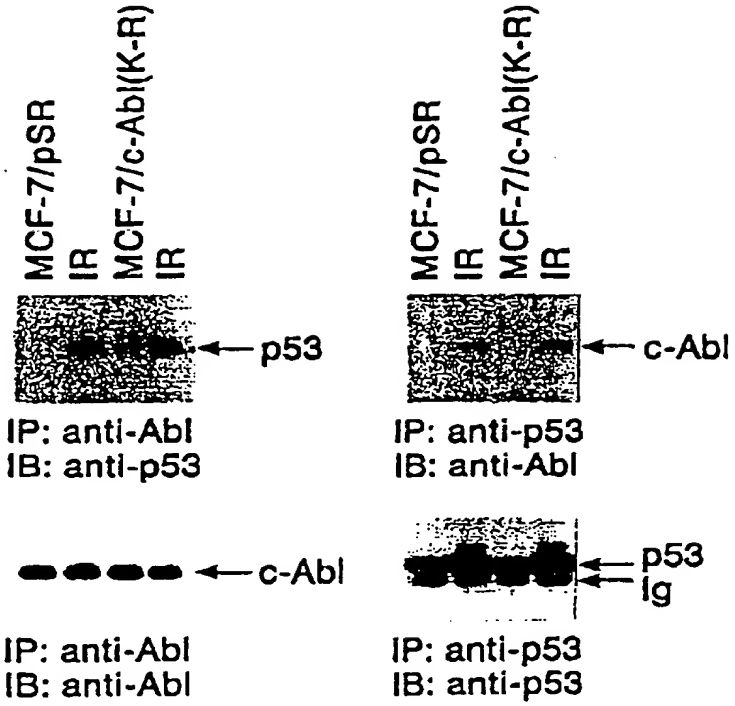


FIG. 3D

FIG. 4A


MCF-7/pSR
IR
MCF-7/c-Abl(K-R)
IR
p53
IP: anti-Abl
IB: anti-p53
c-Abl
IP: anti-Abl
IB: anti-Abl
MCF-7/pSR
IR
MCF-7/c-Abl(K-R)
IR
c-Abl
IP: anti-p53
IB: anti-Abl
p53
Ig
IP: anti-p53
IB: anti-p53

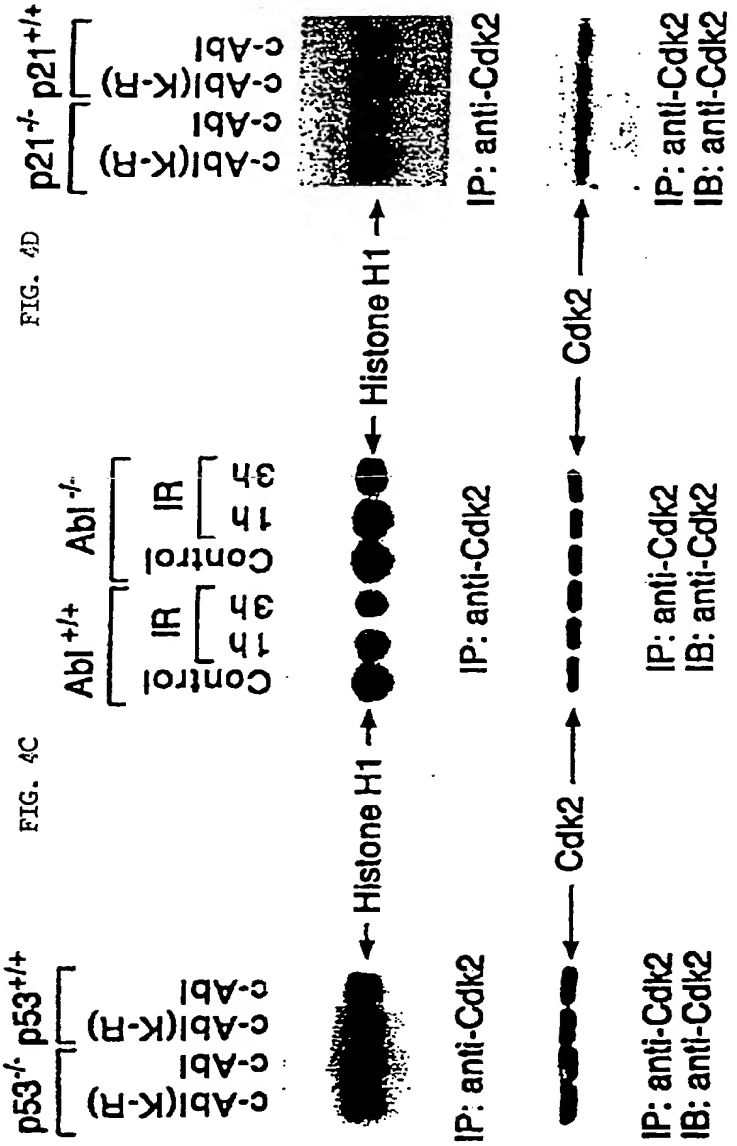


FIG. 4B
p53-/- p53+/+
c-Abl
c-Abl(K-R)
c-Abl
c-Abl(K-R)
Histone H1
IP: anti-Cdk2
Cdk2
IP: anti-Cdk2
IB: anti-Cdk2
FIG. 4C
Abl+/+ Abl-/-
Control
1h
3h
IR
Control
1h
3h
IR
Histone H1
IP: anti-Cdk2
Cdk2
IP: anti-Cdk2
IB: anti-Cdk2
FIG. 4D
p21-/- p21+/+
c-Abl
c-Abl(K-R)
c-Abl
c-Abl(K-R)
IP: anti-Cdk2
IP: anti-Cdk2
IB: anti-Cdk2

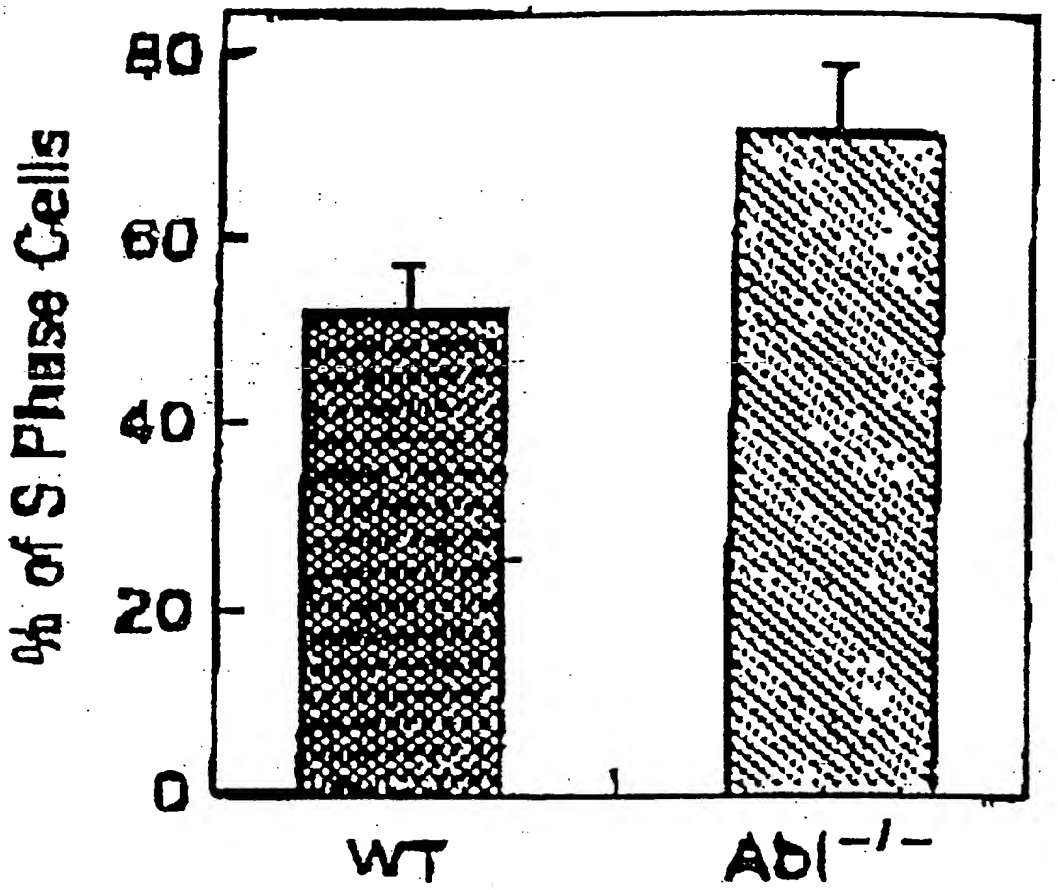


FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/12498

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/17 A61K48/00 G01N33/53 //C07K14/47,C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K G01N C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. GOGA ET AL.: "p53 DEPENDENT GROWTH SUPPRESSION BY THE c-Abl NUCLEAR TYROSINE KINASE." ONCOGENE, vol. 11, no. 4, 17 August 1995, BASINGSTOKE, GB, pages 791-799, XP002046166 cited in the application	1-4,6, 20-23, 38,39
Y	see the whole document	5,7-19, 24-29
	--- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
7 November 1997	17. 12. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/US 97/12498

C.(Continuation) DOCUMENTS CONSIDERED T BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R. SANCHEZ-PRIETO ET AL.: "LACK OF CORRELATION BETWEEN p53 PROTEIN LEVEL AND SENSITIVITY TO DNA-DAMAGING AGENTS IN KERATINOCYTES CARRYING ADENOVIRUS E1a MUTANTS." ONCOGENE, vol. 11, no. 4, 17 August 1995, BASINGSTOKE, GB, pages 675-682, XP002046167	1-4,6, 16-23, 28,29, 38-41
Y	see page 675, abstract see page 680, right-hand column, paragraph 2	5,7-15, 24-27
Y	WO 95 28948 A (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 2 November 1995 see claims	5,7-19, 24-29
X	WO 95 28499 A (DANA-FARBER CANCER INSTITUTE, INC.) 26 October 1995 see page 6, line 8 - page 7, line 19; claims; example VIII	30-37
A	C.L. SAWYERS ET AL.: "THE NUCLEAR TYROSINE KINASE c-Abl NEGATIVELY REGULATES CELL GROWTH." CELL, vol. 77, 8 April 1994, CAMBRIDGE, MA, US, pages 121-131, XP002046168 cited in the application see the whole document	1-41
P,X	Z.-M. YUAN ET AL.: "ROLE FOR c-Abl TYROSINE KINASE IN GROWTH ARREST RESPONSE TO DNA DAMAGE" NATURE, vol. 382, 18 July 1996, LONDON GB, pages 272-274, XP002046169 cited in the application see the whole document	1-4,6, 20-23, 38,39
P,X	Z.-M. YUAN ET AL.: "GENOTOXIC DRUGS INDUCE INTERACTION OF THE c-Abl TYROSINE KINASE AND THE TUMOR SUPPRESSOR PROTEIN p53." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 43, 25 October 1996, MD US, pages 26457-26460, XP002046170 see the whole document	1-4,6, 16-23, 28,29, 38,39

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INTERNATIONAL SEARCH REPORT

Intl ional Application No
PCT/US 97/12498

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	N.L. LILL ET AL.: "BINDING AND MODULATION OF p53 BY p300/CBP COACTIVATORS." NATURE, vol. 387, no. 6635, 19 June 1997, LONDON GB, pages 823-827, XP002046171 see the whole document ---	1-4,6, 20-23, 38-41
P,X	M.L. AVANTAGGIATI ET AL.: "RECRUITMENT OF p300/CBP IN p53-DEPENDENT SIGNAL PATHWAYS." CELL, vol. 89, 27 June 1997, CAMBRIDGE, MA, US, pages 1175-1184, XP002046172 see the whole document -----	1-4,6, 20-23, 38-41

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/12498

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/12498

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 1-19 (as far as being related to an in vivo method) and 20-29 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/US 97/12498

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9528948 A	02-11-95	AU 2392495 A	16-11-95
		EP 0760675 A	12-03-97
		HU 76258 A	28-07-97
		NO 964527 A	17-12-96
		PL 317105 A	17-03-97
		SK 136796 A	09-07-97
WO 9528499 A	26-10-95	US 5658784 A	19-08-97